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[12780/101]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

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In re Application of:	Leonard et al.	
For:	VACCINES FOR MYCOPLASMA BOVIS AND METHODS OF USE	Examiner: Ford Art Unit: 1645
Filed:	November 8, 2000	
Serial No.:	09/708,352	
Customer No.:	26646	

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APPEAL BRIEF TRANSMITTAL

SIR:

Transmitted herewith for filing in the above-identified patent application, please find an Appeal Brief pursuant to 37 C.F.R. § 41.37.

Please charge the small entity Appeal Brief fee of **$250.00**, and any other fees that may be required in connection with this communication to the deposit account of **Kenyon & Kenyon LLP**, deposit account number **11-0600**.

Applicants hereby request a **five-month extension of time** for submitting the Appeal Brief. The extended period for submitting the Appeal Brief expires on May 27, 2006. Please charge the **$1,080.00** small entity extension fee and any other fee that may be required to Deposit Account No. 11-0600. A duplicate of this Transmittal is enclosed.

Respectfully submitted,

Dated: MAY 22, 2006

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SERIAL NO.	:	09/708,352
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APPEAL BRIEF

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Real Party in Interest

The real party in interest for U.S. Patent Application Serial No. 09/708,352 is:

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Biomune Company is a wholly-owned subsidiary of:

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FRANCE

Related Appeals and Interferences

There are no related appeals or interferences.

Status of Claims

Claims 1, 3-12, and 29-56 are pending. Claims 1, 3-12, and 29-56 are under rejection and are being appealed. Claims 2, and 13-28 have been canceled.

Status of Amendments

An Amendment under 37 C.F.R. §1.116 (an Amendment after Final) was filed October 24, 2005, 2005 but was not entered.

Summary of Claimed Subject Matter

The invention defined by independent **claim 1** is a vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species **{specification, page 4, lines 7-8}** comprising at least one inactivated or attenuated *Mycoplasma bovis* biotype **{specification, page 4, lines 8-9}**, an adjuvant **{specification, page 8, lines 7-8}**, and a pharmaceutically acceptable excipient **{specification, page 4, lines 9-10}**, and wherein the adjuvant does not include saponin **{specification, page 8, lines 7-26, particularly line 21}** and the clinical disease includes respiratory pneumonia **{specification, at Example 7, page 21, line 27 to page 22, line 22, and the abstract}**.

The invention defined by independent **claim 5** is a vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species **{specification, page 4, lines 7-8}** comprising at least one inactivated or attenuated *Mycoplasma bovis* biotype **{specification, page 4, lines 8-9}**, an adjuvant **{specification, page 8, lines 7-8}**, and a pharmaceutically acceptable excipient **{specification, page 4, lines 9-10}**, wherein at least one of the inactivated or attenuated *Mycoplasma bovis* biotypes is selected from the group consisting of biotype A **{specification, page 6, line 20}**, biotype B **{specification, page 6, line 20}** and biotype C **{specification, page 6, line 20}**, and wherein the adjuvant does not include saponin **{specification, page 8, lines 7-26, particularly line 21}**.

The invention defined by independent **claim 8** is a vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species **{specification, page 4, lines**

7-8} comprising at least two inactivated or attenuated *Mycoplasma bovis* biotypes **{specification, page 9, lines 1-2}** and a pharmaceutically acceptable excipient **{specification, page 4, lines 9-10}**.

The invention defined by independent **claim 29** is a vaccine which is protective against *Mycoplasma bovis* mastitis in a bovine species **{specification, at Example 5, pages 18-20, particularly page 19, lines 17-32, abstract}** comprising at least one inactivated or attenuated Mycoplasma *bovis* biotype **{specification, page page 4, lines 8-9}** and a pharmaceutically acceptable excipient **{specification, page 4, lines 9-10}**.

The invention defined by independent **claim 52** is a whole-cell vaccine **{specification, page 16, lines 22-28}** which is protective against *Mycoplasma bovis* clinical disease in a bovine species **{specification, page 4, lines 7-8}** comprising at least one inactivated or attenuated *Mycoplasma bovis* biotype **{specification, page 4, lines 8-9}** and an adjuvant selected from the group consisting of: an aluminum hydroxide-oil emulsion; a mineral, vegetable, or fish oil-water emulsion; a water-oil-water emulsion; *E. coli* J5; dextran sulfate; iron oxide; sodium alginate; Bacto-Adjuvant; a synthetic polymer; Carbopol; a poly-amino acid; a co-polymer of amino acids; carrageenan; REGRESSIN®; N, N-dioctadecyl-N'-N'-bis(2-hydroxyethyl) propanediamine; a long chain polydispersed β(1,4) linked mannan polymer interspersed with O-acetylated groups; deproteinized cell wall extracts from a non-pathogenic strain of *Mycobacterium*; mannite monooleate; and paraffin oil **{specification, page 8, lines 16-26 and page 11, lines 3-4}**.

The invention defined by independent **claim 56** is a vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species **{specification, page 4, lines 7-8}** comprising at least one attenuated *Mycoplasma bovis* biotype **{specification, page 4, lines 8-9}** and a pharmaceutically acceptable excipient **{specification, page 4, lines 9-10}**, wherein the clinical disease includes respiratory pneumonia **{specification, at Example 7, page 21, line 27 to page 22, line 22, and the abstract}**.

Grounds of Rejection to be Reviewed on Appeal

The following grounds of rejection are present in this appeal:

(1) claims 1, 3, 5, 6, 29, 30, 40-44, and 52-55 have been rejected as anticipated under 35 U.S.C. §102(b) by Boothby, Immunologic Responses to Mycoplasma bovis, University Microfilm International (Dissertation) 1-172, 1982 (Boothby I);

(2) claims 1, 4, 5, 7, 29, 30, and 56 have been rejected as anticipated under 35 U.S.C. §102(b) by Thorns et al., 1980, Res. Vet. Sci. 29:328-332 (Thorns); and

(3) claims 1, 3-12, and 29-56 have been rejected as obvious under 35 U.S.C. §103(a) over Boothby I in view of Poumarat et al., 1994, Vet. Microbiol. 40:305-321 (Poumarat) and Thorns.

Argument

Ground of rejection 1

Are claims 1, 3, 5, 6, 29, 30, 40-44, and 52-55 anticipated under 35 U.S.C. §102(b) by Boothby, Immunologic Responses to Mycoplasma bovis, University Microfilm International (Dissertation) 1-172, 1982 (Boothby I)?

Claims 1, 3, 5, 6, 29, 30, 40-44, and 52-55 are rejected as being anticipated by Boothby I. These claims do not stand or fall together, but instead should be grouped according to the subheadings below.

Claims 1, 3, 5, 6, 29, 30, 40-44, and 52-55 (all the claims subject to this rejection)

Boothby's vaccine does not anticipate claims 1, 3, 5, 6, 29, 30, 40-44, and 52-55 because there is a clear difference between the Appellants' vaccine and Boothby I's vaccine. Boothby I's vaccine produces a very unfavorable reaction - all of Boothby I's animals showed hypersensitivity (see Boothby I, page 136, 3rd paragraph: "All groups receiving adjuvant preparations developed delayed-type hypersensitivity ...").

In contrast, the presently claimed vaccines do not cause unfavorable reactions. See the specification at page 23, lines 2-3: "No unfavorable reactions resulting from the vaccine's use have been reported;" page 23, lines 14-15: "No unfavorable reactions in animals receiving the product have been reported;" page 20, line 1: "No injection reactions were observed;" and the abstract: "These vaccines demonstrate no undesirable side effects ..."

This is a real difference between the Appellants' vaccine and Boothby I that must be due to the nature of the vaccine, and thus indicates that the vaccine of Boothby I does not anticipate the presently claimed vaccine.

Claims 5, 6, and 54

Claim 5 and dependent claims 6 and 54 require that the vaccine comprises particular biotypes that are not disclosed in Boothby I. Claims 5, 6, and 54 each require at least one biotype selected from the group consisting of biotype A, biotype B and biotype C. Boothby I does not disclose biotype A, B, or C. Thus, Boothby I cannot anticipate these claims.

Claims 29, 30, and 40-44

Claims 29, 30, and 40-44 all contain the limitation that the claimed vaccine must be "protective against *Mycoplasma bovis* mastitis in a bovine species." The Examiner argued that this limitation is merely an "intended use" and therefore is not sufficient to avoid anticipation by Boothby I. See the Office Action, dated May 25, 2005, page 4, line 17 to page 5, line 12.

The Appellants do not agree. Being protective against mastitis is not simply an intended use but rather is a functional characteristic of the vaccine itself. The characteristic of being protective against mastitis distinguishes the claims over the prior art, such as the vaccine disclosed in Boothby I. The evidence of record demonstrates that prior art vaccines, such as Boothby I's, were not protective against mastitis. Persons skilled in the art, having knowledge of Boothby I and other prior art, did not view the then-existing vaccines as being protective against mastitis.

For example, Heller et al., 1993, Vet. Microbiol. 37:127-133 (Heller), when referring to methods of controlling the spread of *Mycoplasma bovis*-caused mastitis, did not mention that one should vaccinate to control mastitis but instead stated that culling is necessary. See page 127: "To control the spread of this disease, an early detection of

the pathogen is crucial since the removal and culling of infected cows is necessary to prevent fresh infections."

Hanson, (September, 2001) Bovine Veterinarian 4-8 (Hanson I) and Hanson, (October, 2001) Bovine Veterinarian 12-20 (Hanson II), described methods to prevent mastitis or mitigate its effects, but the methods do not include vaccination, indicating that no vaccine protective against mastitis was known to the art. This failure to mention vaccination is telling, since there clearly was recognition in the art that *Mycoplasma bovis*-caused mastitis was a serious problem. For example, Hanson I, at page 4, quotes a veterinarian as follows:

> "*Mycoplasma* mastitis is a doubly insulting disease," says Blackmer. "Not only can it be remarkably contagious when it is present but it absolutely does not respond to antibiotic therapy. In fact, treatment can actually cause epidemics, because it frequently is spread by unsound intramammary therapy practices."

If vaccination had been available to combat a problem as serious as *M. bovis*-caused mastitis, Heller, Hanson I, and Hanson II would have been expected to mention it, but they did not.

The Office Action dated May 25, 2005, page 5, lines 5-7, refused to consider this evidence, stating: "Applicant's referral to other publications (Heller et al, 1993, Hanson, September 2001 and Hanson, October 2001) to support their position is irrelevant since Boothby teach the claimed vaccine compositions." [emphasis added] However, this misunderstands the import of the evidence. Heller and the two Hanson publications demonstrate that Boothby I does not teach the claimed vaccines. The Appellants submit that the Office Action has assumed the issue to be decided - whether Boothby I discloses the claimed vaccines - before considering all the evidence that should be used to decide that issue. The U.S. Patent & Trademark Office has the burden of proving a case of anticipation, based upon reasoned arguments, after considering all relevant evidence. The Appellants submit that this has not been done.

The Office Action did not explain why, if Boothby I provided a vaccine against mastitis, the art was still recommending culling and other non-vaccine approaches as the only methods of combating mastitis nearly twenty years after Boothby I's disclosure became public.[1] Based on the record as it currently stands, the inevitable conclusion is that Boothby I's vaccine was not protective against mastitis, and thus could not have been the same as the claimed vaccine.

The Office Action dated May 25, 2005 cited *In re Casey*, 370 F. 2d 576, 152 USPQ 235 (CCPA 1967) and *In re Otto*, 312 F. 2d 937, 136 USPQ 458 (CCPA 1963) in support of its position with respect to the limitation of protection against mastitis.

Casey is not applicable to the present fact pattern because the functional properties of the claimed device in *Casey* were found to be inherently disclosed in the Kienzle prior art reference. See 370 F. 2d at 941, 152 U.S.P.Q. at 238, where the Court of Customs and Patent Appeals agreed with the reasoning of the Board of Appeals and stated: "The rationale of the board clearly deducible from the language employed is that the Kienzle apparatus as it obviously must be constructed would inherently perform all of the functions called for in claim 1 ..." In the present application, the functional property of being protective against mastitis is not found in the prior art, either explicitly or inherently.

In *Otto*, the claims were rejected for obviousness over a large number of references that collectively disclosed all the limitations recited in the claims. That is not the case here, where the record contains no prior art, either alone or in combination, disclosing the limitation of "protective against bovine mastitis." Instead, the record contains compelling evidence that the prior art lacked this limitation.

---

[1] Boothby I is dated 1982. Heller is dated 1993. Hanson I and Hanson II are dated 2001.

A much more recent decision, in the Federal Circuit, which specifically dealt with functional characteristics of product claims, and which is therefore particularly applicable to the present application is *Union Oil Co. of Cal. v. Atlantic Richfield Co.*, 208 F. 3d 989, 54 U.S.P.Q. 2d 1227 (Fed. Cir. 2000). In *Union Oil*, the claims at issue were directed to gasolines that were defined as being suitable for combustion in automotive engines. See 208 F. 3d at 995, 54 U.S.P.Q. 2d at 1231:

> The claims of the '393 patent recite either "[a]n unleaded gasoline suitable for combustion in an automotive engine" or "[a]n unleaded gasoline fuel suitable for combustion in a spark ignition automotive engine."

The district court construed these claims to cover only automotive fuels, not also aviation or racing fuels. This was primarily due to the specification's teaching that the functional characteristic of being suitable for use in automotive engines addressed the problem the invention was directed toward, together with the claims' recitation of that characteristic. This resulted in a finding that the quoted functional language was a real limitation of the claims, and not just an intended use. See 208 F. 3d at 995-996, 54 U.S.P.Q. 2d at 1231-1232:

> The district court's interpretation also finds extensive support in the specification. The patentees described the problem that their invention addressed:
>
> > One of the major environmental problems confronting the United States and other countries is atmospheric pollution (i.e., "smog") caused by the emission of gaseous pollutants in the exhaust gases from automobiles. This problem is especially acute in major metropolitan areas, such as Los Angeles, Calif., where the atmospheric conditions and the great number of automobiles account for aggravated air pollution.
>
> ...
>
> The patentees tailored their research and their patent to ordinary fuels for use in standard passenger cars. Thus, the claim language, further informed by the specification, shows that the district court correctly read the claims to cover ordinary automotive fuel.

> Because the '393 patent covers only standard automotive fuel, the district court correctly determined that specialty fuels within other limitations of the claims do not anticipate under 35 U.S.C. § 102.

As in *Union Oil*, claims 29, 30, and 40-44 of the present application recite the functional characteristic at issue - "protective against *Mycoplasma bovis* mastitis." Also as in *Union Oil*, the present specification stresses the problem of mastitis and teaches that the vaccines of the present invention address that problem by providing actual data showing the vaccines to be protective against mastitis. See the specification, page 2, lines 1-11:

> Diseases caused by mycoplasmas are often resistant to antimicrobial therapy, leaving no effective means of treatment. Consequently, the only effective control method is to cull animals from a herd. This has enormous economic implications in the dairy industry where losses are measured by the value of the culled animals as well as the impact on both milk quality and quantity due to clinical and subclinical infections. Mycoplasma infections resulting in bovine mastitis are increasing in prevalence and geographical distribution. In the United States, this higher prevalence is due to a larger and-more intense cattle production industry in which herds are rapidly expanding, placing them at greater risk. Increased incidence of *M. bovis* infection and related infectious disease in dairy herds has been noted worldwide (Jasper, DE 1982, J. Amer. Vet. Med. Assn. 181:158-162).

See also Example 5, pages 18-20, where a large decrease in the number of mastitis cases occurred in a herd that was vaccinated with the vaccine of the present invention. See in particular page 19, lines 17-32:

> Comparative results were used to measure efficacy of the vaccine. Samples taken from all animals presenting with clinical mastitis were cultured by an independent laboratory to monitor the absence or presence of Mycoplasma bovis infection of the mammary gland. Field evaluations were made by comparing clinical incidence of mastitis caused by Mycoplasma bovis following herd vaccination to the base line herd incidence prior to vaccination. Results were as follows:
>
> Pre Vaccination Base Line Incidence:
> 155 confirmed positive clinical Mycoplasma bovis infections
>
> Post Vaccination Herd Incidence:
> 1st year following vaccination:
> 24 confirmed positive clinical Mycoplasma bovis infections
> 2nd year following vaccination:
> 1 confirmed positive clinical Mycoplasma bovis infection.

In view of the Federal Circuit's guidance in *Union Oil* as to how such a claim recitation should be construed, the recitation of "protective against *Mycoplasma bovis* mastitis" is a true limitation of claim 29, 30, and 40-44 and serves to distinguish these claims over the prior art.

Even if the recitation of "protective against bovine mastitis" is viewed as an intended use, this rejection should be withdrawn. In connection with the interpretation of this recitation as an intended use, the Office Action cited *Casey* and *Otto* as support for the proposition that (sentence bridging pages 4 and 5): "If the prior art is capable of performing the intended use, then it meets the claim." This proposition is inapplicable here, because the evidence of record shows that the prior art is not capable of protecting against mastitis.

Where the prior art product was not capable of performing the intended use, the Board of Patent Appeals and Interference held that claims reciting the intended use were not anticipated. In *Ex parte Hervy A. Morris* (available at 1998 WL 1736155), a claim directed to a cutting device recited "to deflect the liquid jet stream when the cutting element is moved to the idle position." The Examiner interpreted this recitation as an intended use and rejected the claim over *Casey* and *Otto*, stating: "If the prior art structure is capable of performing the intended use, then it meets the claim."

> [t]he phrase "to deflect the liquid-jet stream ....." should not be construed as defining structure. It does not describe any structure; it merely expresses what the disk is desired to do. However, it has well been established that, a recitation of the intended use of the claimed invention must result in a structural difference between the claimed invention and the prior art in order to patentably distinguish the claimed invention from the prior art. If the prior art structure is capable of performing the intended use, then it meets the claim. In re Casey, [370 F.2d 576, 580,] 152 USPQ 235[[, 238] (CCPA 1967); In re Otto, [312 F.2d 937, 940,] 136 USPQ 458, 459 (CCPA 1963).

Ex parte Hervy A. Morris, page 2.

The Board of Patent Appeals and Interference reversed this rejection, stating:

> Although we appreciate the examiner's position, we do not agree with his argument, because in our view the disk 620 of Driver [the prior art relied on by the Examiner] is not capable of performing the intended use recited, i.e., of "deflect[ing] the liquid-jet stream when the cutting element is moved to the idle position."

Ex parte Hervy A. Morris, page 2.

The evidence of record shows that Boothby I's vaccines were not "capable of performing the intended use" because the evidence of record shows that Boothby I's vaccines were not protective against mastitis. Accordingly, *Casey* and *Otto* are not applicable and claims 29, 30, and 40-44 are not anticipated by Boothby I.

Claims 52 and 55

Claims 52 and 55 recite that the vaccine comprises an adjuvant selected from a group that does not include the adjuvants listed in Boothby I.[2] Therefore, Boothby I does not anticipate claims 52 and 55.

---

[2] At page 131, Boothby I discloses the use of the following adjuvants:
Freund's incomplete adjuvant
N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP)
Amphotericin B
Combined magnesium/aluminum hydroxide
Killed *Bordatella pertussis*

Ground of rejection 2

Are claims 1, 4, 5, 7, 29, 30, and 56 anticipated under 35 U.S.C. §102(b) by Thorns et al., 1980, Res. Vet. Sci. 29:328-332 (Thorns)?

Claims 1, 4, 5, 7, 29, 30, and 56 are rejected as being anticipated by Thorns. These claims do not stand or fall together, but instead should be grouped according to the subheadings below.

Claims 1, 4, 5, 7, 29, 30, and 56 (all of the claims subject to this rejection)

There is a clear difference between the presently claimed vaccines and Thorn's mycoplasma strains. The presently claimed vaccines do not cause unfavorable reactions. See the specification at page 23, lines 2-3: "No unfavorable reactions resulting from the vaccine's use have been reported;" page 23, lines 14-15: "No unfavorable reactions in animals receiving the product have been reported;" page 20, line 1: "No injection reactions were observed;" and the abstract: "These vaccines demonstrate no undesirable side effects ..."

All of the strains in Thorns caused some kind of histopathological change. See the right column in Table 1 on page 329, which shows that only the control (i.e., no *Mycoplasma bovis*) injections resulted in no histopathological changes.

Claims 1, 4, 5, 7, 29, 30, and 56 all recite "vaccines" that are "protective" against diseases caused by *Mycoplasma bovis* in bovines. Thorns does not even disclose a vaccine. Thorns discloses only attenuated strains of *Mycoplasma bovis* that were injected into mice. There is no disclosure in Thorns that the attenuated strains were protective against any disease in the injected mice, and certainly not against any disease

in bovines. Thorns does not even disclose any data that indicate the attenuated strains caused any stimulation of the immune systems of the mice against *Mycoplasma bovis.*

Thorns showed that highly passaged strains were attenuated in the sense that the highly passaged strains themselves did not cause responses such as inflammation or abnormal glands to the same degree as low passaged strains. Thus, Thorns disclosed attenuated *Mycoplasma bovis* strains. But claims 1, 4, 5, 7, 29, 30, and 56 are not directed simply to attenuated strains. They are directed to attenuated strains that are capable of functioning as vaccines. Thorns contains no evidence that the attenuated strains disclosed therein could function as vaccines, to protect against disease caused by later exposure to *Mycoplasma bovis*. In particular, Thorns provided no evidence that the mice that were given the attenuated strains were protected from disease when later challenged with *Mycoplasma bovis*. Apparently, Thorns did not even challenge the mice.

The Office Action concluded that, since Thorns's highly attenuated strains did not cause disease themselves, they must have been able to protect against disease, i.e., that the attenuated strains were vaccines. See the Office Action dated May 25, 2005, page 6, lines 2-6:

> Thorns et al teach that all mice that were inoculated with *M. bovis* passaged over 91 times had normal glands and showed not signs of systematic response (page 329, Table 1). Therefore, the mice vaccinated with *M. bovis* passaged over 91 times appeared to be protected against systematic response.

But this logic is fundamentally flawed. It confuses one characteristic - the lack of ability to cause disease - with another, not necessarily related, characteristic - the ability to protect against disease. The Office Action provided no evidence that an attenuated

strain having the former characteristic would necessarily have the second characteristic as well.

Moreover, the authors of Thorns stated that their strains were not vaccines. The authors considered that the work they disclosed only provided information and a starting point for research that might someday "perhaps" lead to the production of a vaccine against *Mycoplasma bovis*. In view of this statement, the strains described in Thorns could not already be vaccines. See page 332, right column, 3rd paragraph:

> Whatever mechanisms the virulent strains have lost or modified, they should provide further insight into the pathogenesis of *M. bovis* mastitis which could perhaps lead to a stable vaccine for this disease. [emphasis added]

Claims 1, 4, 5, and 7

Claims 1, 4, 5, and 7 recite "an adjuvant." Thorns does not disclose an adjuvant. For this reason, Thorns does not anticipate claims 1, 4, 5, and 7.

Claims 29, 30, and 56

Claims 29, 30, and 56 recite the limitations that the claimed vaccines must be "protective against *Mycoplasma bovis* mastitis" (claims 29 and 30) or "protective against *Mycoplasma bovis* clinical disease ... wherein the clinical disease includes respiratory pneumonia" (claim 56). As discussed above in connection with the rejection over Boothby I, these recitations are not simply an "intended use" but instead are functional limitations that confer patentable distinction on the claims. Thorns contains no showing that the attenuated strains disclosed therein are capable of protecting against any diseases. Thus, for this reason as well, Thorns does not anticipate claims 29, 30, and 56.

Ground of rejection 3

Are claims 1, 3-12, and 29-56 obvious over Boothby I in view of Poumarat et al., 1994, Vet. Microbiol. 40:305-321 (Poumarat) and Thorns?

Claims 1, 3-12, and 29-56 have been rejected as being obvious over Boothby I in view of Poumarat et al., 1994, Vet. Microbiol. 40:305-321 (Poumarat) and Thorns. These claims do not stand or fall together, but instead should be grouped according to the subheadings below.

Claims 1, 3-12, and 29-56 (all of the claims subject to this rejection)

As discussed above, the presently claimed vaccine does not cause unfavorable reactions. As discussed above, this limitation is lacking in Boothby I and Thorns, since the *M. bovis* in Boothby I caused hypersensitivity and the *M. bovis* in Thorns caused histopathological changes. Thus, these two publications lack a disclosure of this claim limitation.

As explained more fully below, Poumarat did not disclose vaccines of any kind, and thus failed to teach or suggest a vaccine that does not cause unfavorable reactions.

In view of the complete lack of disclosure of this limitation in the prior art, no combination of the cited references can possibly disclose or suggest this limitation, and the Appellants thus submit that a *prima facie* case of obviousness for claims 1, 3-12, and 29-56 has not been and cannot be made.

Claims 8-12, 31-39, and 46-51

Claims 8-12, 31-39, and 46-51 recite "at least two" *M. bovis* biotypes.

Boothby I does not disclose a vaccine that contains more than one biotype. Even if Thorns is viewed as disclosing vaccines (which the Appellants dispute), Thorns still does not disclose a vaccine containing more than one biotype since all the strains in Thorns were administered individually.

Poumarat does not disclose any vaccines since Poumarat is limited to a study of the antigenic characteristics of certain strains of *Mycoplasma bovis*. Moreover, Poumarat discourages, and thus teaches away from, the use of more than one biotype.

Poumarat divided *Mycoplasma bovis* isolates into 13 different "genomic groups." Poumarat then looked at the antigenic variability between and among these genomic groups. Although Poumarat found much antigenic variability, this variability did not correlate with membership in any particular genomic group. In other words, the same amount of antigenic variability could be found within groups as between groups. See page 318, 2nd paragraph:

> Antigenic profiles of the *M. bovis* strains obtained by immunoblotting with J008 calf serum differed markedly one from the other, the heterogeneity being equally great among strains belonging to the same genomic group and those coming from different genomic groups. There appeared to be no relation between the genomic variability of *M. bovis* and the antigenic variability ...

Because Poumarat teaches that antigenic variability is as great within *Mycoplasma bovis* groups as across *Mycoplasma bovis* groups, Poumarat teaches that there would be no gain in antigenic variability from including more than one type of *Mycoplasma bovis* in a vaccine. That is, there would be no point in having more than one type of *Mycoplasma bovis* in a vaccine. Poumarat thus discourages one of ordinary skill in the art from including more than one biotype in a vaccine, and thus teaches away from the invention defined by claims 8-12, 31-39, and 46-51.

"A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant." *Monarch Knitting Mach. Corp. v. Sulzer Morat GmbH*, 139 F.3d 877, 885, 45 USPQ2d 1977, 1984 (Fed. Cir. 1998).

Claims 34-39 and 46-51

Poumarat's teaching away from a vaccine containing more than one biotype is especially pertinent in connection with claims 34-39 and 46-51. These claims all require that the at least two biotypes be genetically different, as judged by analysis of DNA or RNA. Poumarat expressly teaches that such genetic differences are irrelevant with respect to antigenicity since Poumarat teaches that there appears to be "no relation between the genomic variability of *M. bovis* and the antigenic variability." One of ordinary skill in the art would clearly interpret this conclusion as a teaching that nothing is to be gained from including biotypes that are genetically different in a vaccine, and thus would be discouraged from the invention of claims 34-39 and 46-51. Such a distinct teaching away from the Appellants' invention in the prior art constitutes a strong indication of non-obviousness, and *a fortiori* negates any possible case of *prima facie* obviousness.

Claims 29, 30, and 40-45

Claims 29, 30, and 40-45 recite that the vaccine is "protective against *Mycoplasma bovis* mastitis."

None of Boothby I, Thorns, or Poumarat disclose or suggest this limitation.

Furthermore, there was a long-felt need in the art for an effective vaccine against bovine mastitis. See, e.g., Hanson, (September, 2001) Bovine Veterinarian 4-8 (Hanson I) and Hanson, (October, 2001) Bovine Veterinarian 12-20 (Hanson II), which contain extensive descriptions of the problems caused by bovine mastitis and the difficulty of dealing with this disease. For example, Hanson I quotes a veterinarian as follows (page 4):

> "*Mycoplasma* mastitis is a doubly insulting disease," says Blackmer. "Not only can it be remarkably contagious when it is present but it absolutely does not respond to antibiotic therapy. In fact, treatment can actually cause epidemics, because it frequently is spread by unsound intramammary therapy practices."

The art also discloses that others tried and failed to produce a vaccine protective against mastitis. Boothby et al., 1986, Can. J. Vet. Res. 50:200-204 (Boothby II) shows this failure of others, and also teaches away from the present claims. Boothby II tested whether killed *M. bovis* would be effective as a vaccine against bovine mastitis and found that it was not. Despite their prior exposure to killed *M. bovis*, the treated cows in Boothby II were not protected against infection (see page 202, middle column: "All experimentally challenged quarters became infected ..."). Thus, Boothby II was unsuccessful. Such a failure is a clear and strong deterrent to others. The skilled person therefore would undoubtedly have been deterred and discouraged by Boothby II from attempting to produce *M. bovis* vaccine, and thus would not even have sought the solution provided by the Appellants.

Moreover, the treated animals in Boothby II showed poorer milk production than the untreated animals. The treated cows suffered significant and persistent reductions in the level of milk production. The control cows exhibited a smaller and more transient drop in milk production. See Figure 2 on page 202 for a comparison of treated and control cows. Thus, not only did the killed *M. bovis* fail to protect the treated cows, but

it caused milk production to be even worse than it would have been had the cows not been treated. Since the primary purpose for having dairy herds is to produce milk, one of ordinary skill in the art would certainly be deterred by a result that decreased the production of milk.[3] Given that Boothby would have deterred the skilled person in two major respects - lack of efficacy and decrease in milk production - Boothby must be seen as teaching away from the Appellants' invention.

Claim 56

Claim 56 is directed to attenuated vaccines that are protective against respiratory pneumonia.

Boothby I and Poumarat do not disclose attenuated *Mycoplasma bovis*. As discussed above, although Thorns does disclose attenuated strains of *Mycoplasma bovis,* Thorns states that these strains are not vaccines, but might provide "further insight" which could "perhaps" lead to the development of a vaccine. See Thorns, page 332, right column, 3rd paragraph:

> Whatever mechanisms the virulent strains have lost or modified, they should provide further insight into the pathogenesis of *M. bovis* mastitis which could perhaps lead to a stable vaccine for this disease. [emphasis added]

Given the lack of disclosure of an attenuated vaccine that is protective against respiratory pneumonia in any of Boothby I, Thorns, or Poumarat, and the lack of any suggestion as to how such a vaccine could be produced in those references, it cannot properly be said that those references make obvious claim 56.

---

[3] This is recognized by Boothby II at page 200, right column, where it is stated: "If prophylactic vaccination is to be efficacious, it must have minimal effects on the health and productive capabilities of

CONCLUSION

For the reasons discussed above, the Appellants respectfully request that the Board of Patent Appeals and Interferences reverse:

(1) the rejection of claims 1, 3, 5, 6, 29, 30, 40-44, and 52-55 as anticipated under 35 U.S.C. §102(b) by Boothby, Immunologic Responses to Mycoplasma bovis, University Microfilm International (Dissertation) 1-172, 1982 (Boothby I);

(2) the rejection of claims 1, 4, 5, 7, 29, 30, and 56 as anticipated under 35 U.S.C. §102(b) by Thorns et al., 1980, Res. Vet. Sci. 29:328-332 (Thorns); and

(3) the rejection of claims 1, 3-12, and 29-56 as obvious under 35 U.S.C. §103(a) over Boothby I in view of Poumarat et al., 1994, Vet. Microbiol. 40:305-321 (Poumarat) and Thorns.

Respectfully submitted,

Dated: MAY 22, 2006

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the cow."

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CLAIMS APPENDIX

1. A vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species comprising at least one inactivated or attenuated *Mycoplasma bovis* biotype, an adjuvant, and a pharmaceutically acceptable excipient, and wherein the adjuvant does not include saponin and the clinical disease includes respiratory pneumonia.

2. canceled

3. The vaccine of claim 1, wherein the *Mycoplasma bovis* biotype is inactivated and the amount of each inactivated biotype is at least 10^8 *M.* bovis cells.

4. The vaccine of claim 1, wherein the *Mycoplasma bovis* biotype is attenuated and the amount of each attenuated biotype is at least 10^5 *M. bovis* cells.

5. A vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species comprising at least one inactivated or attenuated *Mycoplasma bovis* biotype, an adjuvant, and a pharmaceutically acceptable excipient, wherein at least one of the inactivated or attenuated *Mycoplasma bovis* biotypes is selected from the group consisting of biotype A, biotype B and Biotype C, and wherein the adjuvant does not include saponin.

6. The vaccine of claim 5, wherein the *Mycoplasma bovis* biotype is inactivated and the amount of each selected inactivated *Mycoplasma bovis* biotype is at least 10^8 *M. bovis* cells.

7. The vaccine of claim 5, wherein the *Mycoplasma bovis* biotype is attenuated and the amount of each selected attenuated *Mycoplasma bovis* biotype is at least 10^5 *M. bovis* cells.

8. A vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species comprising at least two inactivated or attenuated *Mycoplasma bovis* biotypes and a pharmaceutically acceptable excipient.

9. The vaccine of claim 8, further comprising a suitable adjuvant.

10. The vaccine of claim 8, wherein the *Mycoplasma bovis* biotype is inactivated and the amount of each inactivated biotype is at least 10^8 *M. bovis* cells.

11. The vaccine of claim 8, wherein the *Mycoplasma bovis* biotype is attenuated and the amount of each attenuated biotype is at least 10^5 *M. bovis* cells.

12. The vaccine of claim 8, wherein the *Mycoplasma bovis* biotypes are selected from the group consisting of biotype A, biotype B and biotype C.

13-28. (canceled)

29. A vaccine which is protective against *Mycoplasma bovis* mastitis in a bovine species comprising at least one inactivated or attenuated Mycoplasma *bovis* biotype and a pharmaceutically acceptable excipient.

30. The vaccine of claim 29, where the vaccine is protective against *Mycoplasma bovis* mastitis in a bovine species following systemic administration.

31. The vaccine of claim 30, comprising at least two inactivated *Mycoplasma bovis* biotypes.

32. The vaccine of claim 31, wherein the vaccine includes at least one inactivated *Mycoplasma bovis* biotype selected from the group consisting of biotype A, biotype B and biotype C.

33. The vaccine of claim 31 wherein the vaccine contains approximately 10^8 cells of each biotype in a volume of 2-5 milliliters.

34. The vaccine of claim 8 wherein the at least two inactivated or attenuated *Mycoplasma bovis* biotypes are genetically different as determined by an analysis of DNA or RNA from the biotypes.

35. The vaccine of claim 34 wherein the analysis is by PCR fingerprinting, analysis of ribosomal RNA, or analysis of DNA polymorphisms.

36. The vaccine of claim 35 wherein the analysis is by PCR fingerprinting.

37. The vaccine of claim 36 wherein the PCR fingerprinting uses arbitrarily chosen primers.

38. The vaccine of claim 37 wherein the PCR fingerprinting uses as primers 5' NNN NCG NCG NCA TCN GGC 3' (SEQ ID NO:1) and 5' NCG NCT TAT CNG GCC TAC 3' (SEQ ID NO:2).

39. The vaccine of claim 8 wherein the at least two *Mycoplasma bovis* biotypes have been identified as being different biotypes by a process comprising:
(a) isolating DNA from the biotypes;
(b) amplifying the DNA by PCR;
(c) separating the amplified DNA by gel electrophoresis; and
(d) comparing the resulting patterns from the gel electrophoresis to identify the different biotypes.

40. The vaccine of claim 30 wherein, when the vaccine is administered to a plurality of cows in a herd of cows, the incidence of mastitis caused by *Mycoplasma bovis* in the herd before administering is greater than the incidence of mastitis caused by *Mycoplasma bovis* in the herd after administering.

41. The vaccine of claim 40 wherein the vaccine is administered to at least about 50% of the herd.

42. The vaccine of claim 41 where the vaccine is administered together with an adjuvant.

43. The vaccine of claim 42 wherein the adjuvant is an aluminum hydroxide-oil emulsion; a mineral, vegetable, or fish oil-water emulsion; a water-oil-water emulsion; incomplete Freund's adjuvant; *E. coli* J5; dextran sulfate; iron oxide; sodium alginate; Bacto-Adjuvant; a synthetic polymer; Carbopol; a poly-amino acid; a co-polymer of amino acids; saponin; carrageenan; REGRESSIN®; N, N-dioctadecyl-N'-N'-bis(2-hydroxyethyl) propanediamine; a long chain polydispersed β(1,4) linked mannan polymer interspersed with O-acetylated groups; deproteinized cell wall extracts from a non-pathogenic strain of *Mycobacterium*; mannite monooleate; paraffin oil; or muramyl dipeptide.

44. The vaccine of claim 30 where the *Mycoplasma bovis* biotype is inactivated and has been inactivated by treatment with: formalin, azide, freeze-thawing, sonication, heat, sudden pressure drop, detergent, lysozyme, phenol, proteolytic enzymes, β-propiolactone, Thimerosal, or binary ethyleneimine.

45. The vaccine of claim 44 where the *Mycoplasma bovis* biotype has been inactivated by treatment with β-propiolactone.

46. The vaccine of claim 31 wherein the at least two inactivated *Mycoplasma bovis* biotypes are genetically different as determined by an analysis of DNA or RNA from the biotypes.

47. The vaccine of claim 46 wherein the analysis is by PCR fingerprinting, analysis of ribosomal RNA, or analysis of DNA polymorphisms.

48. The vaccine of claim 47 wherein the analysis is by PCR fingerprinting.

49. The vaccine of claim 48 wherein the PCR fingerprinting uses arbitrarily chosen primers.

50. The vaccine of claim 49 wherein the PCR fingerprinting uses as primers 5' NNN NCG NCG NCA TCN GGC 3' (SEQ ID NO:1) and 5' NCG NCT TAT CNG GCC TAC 3' (SEQ ID NO:2).

51. The vaccine of claim 31 wherein the at least two *Mycoplasma bovis* biotypes have been identified as being different biotypes by a process comprising:
(a) isolating DNA from the biotypes;
(b) amplifying the DNA by PCR;
(c) separating the amplified DNA by gel electrophoresis; and
(d) comparing the resulting patterns from the gel electrophoresis to identify the different biotypes.

52. A whole-cell vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species comprising at least one inactivated or attenuated *Mycoplasma bovis* biotype and an adjuvant selected from the group consisting of: an aluminum hydroxide-oil emulsion; a mineral, vegetable, or fish oil-water emulsion; a water-oil-water emulsion; *E. coli* J5; dextran sulfate; iron oxide; sodium alginate; Bacto-Adjuvant; a synthetic polymer; Carbopol; a poly-amino acid; a co-polymer of amino acids; carrageenan; REGRESSIN®; N, N-dioctadecyl-N'-N'-bis(2-hydroxyethyl) propanediamine; a long chain polydispersed β(1,4) linked mannan polymer interspersed with O-acetylated groups; deproteinized cell wall extracts from a non-pathogenic strain of *Mycobacterium*; mannite monooleate; and paraffin oil.

53. The vaccine of claim 1, wherein the *Mycoplasma bovis* biotype is inactivated.

54. The vaccine of claim 5, wherein the *Mycoplasma bovis* biotype is inactivated.

55. The vaccine of claim 52, wherein the *Mycoplasma bovis* biotype is inactivated.

56. A vaccine which is protective against *Mycoplasma bovis* clinical disease in a bovine species comprising at least one attenuated *Mycoplasma bovis* biotype and a

pharmaceutically acceptable excipient, wherein the clinical disease includes respiratory pneumonia.

Evidence Appendix

The evidence relied upon, and where in the record that evidence was entered, is as follows:

1. Boothby, Immunologic Responses to Mycoplasma bovis, University Microfilm International (Dissertation) 1-172, 1982 (Boothby I). Boothby I was applied by the Examiner in an anticipation rejection in the Office Action dated May 25, 2005, bottom of page 2 to middle of page 5.
2. Heller et al., 1993, Vet. Microbiol. 37:127-133 (Heller). Heller was submitted in an Information Disclosure Statement filed April 16, 2002. The Examiner returned a copy of the PTO-1449 Form accompanying this Information Disclosure Statement, with the entry for this publication initialed, with the Office Action dated September 30, 2003.
3. Hanson, (September, 2001) Bovine Veterinarian 4-8 (Hanson I). Hanson I was submitted in an Information Disclosure Statement filed April 16, 2002. The Examiner returned a copy of the PTO-1449 Form accompanying this Information Disclosure Statement, with the entry for this publication initialed, with the Office Action dated September 30, 2003.
4. Hanson, (October, 2001) Bovine Veterinarian 12-20 (Hanson II). Hanson II was submitted in an Information Disclosure Statement filed April 16, 2002. The Examiner returned a copy of the PTO-1449 Form accompanying this Information Disclosure Statement, with the entry for this publication initialed, with the Office Action dated September 30, 2003.

5. *Ex parte Hervy A. Morris* (available in the Westlaw database at 1998 WL 1736155), a copy of which is enclosed herewith since this is a decision of the Board of Patent Appeals & Interferences that has not been published in a West reporter or in United States Patents Quarterly.

6. Thorns et al., 1980, Res. Vet. Sci. 29:328-332 (Thorns). Thorns was applied by the Examiner in an anticipation rejection in the Office Action dated May 25, 2005, middle of page 5 to middle of page 7.

7. Poumarat et al., 1994, Vet. Microbiol. 40:305-321 (Poumarat). Poumarat was applied by the Examiner in an anticipation rejection in the Office Action dated May 25, 2005, middle of page 7 to top of page 12.

8. Boothby et al., 1986, Can. J. Vet. Res. 50:200-204 (Boothby II). Boothby II was submitted in an Information Disclosure Statement filed April 16, 2002. The Examiner returned a copy of the PTO-1449 Form accompanying this Information Disclosure Statement, with the entry for this publication initialed, with the Office Action dated September 30, 2003.

Related Proceedings Appendix

(none)

Immunologic Responses to *Mycoplasma bovis*

By

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A.B. (University of California, Berkeley) 1970
M.S. (University of California, Davis) 1978

DISSERTATION

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Boothby, John Thayer

IMMUNOLOGIC RESPONSES TO MYCOPLASMA BOVIS

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GENERAL INTRODUCTION

Bovine mycoplasmal diseases were unrecognized in the United States from 1892, when eradication of contagious bovine pleuropneumonia (due to Mycoplasma mycoides var mycoides) was complete,[1] until 1962 when mycoplasmal mastitis was first reported in this country.[2] The mycoplasma responsible for this outbreak was M. bovis (formerly M. agalactiae subs bovis).[3] This organism has since been isolated in this country and elsewhere from a variety of anatomical sites and has been associated with respiratory,[4-8] reproductive[9] and joint disease[10-12] as well as mastitis. Apart from M. mycoides var mycoides, M. bovis is probably the most pathogenic mycoplasma isolated from cattle.[13]

Assessment of the precise role of M. bovis in natural bovine disease is confounded because the organism is often isolated from healthy as well as diseased animals.[13-15] The pathogenicity of M. bovis has been shown for a number of anatomical sites under controlled experimental conditions.

Mycoplasma bovis is the most common and most severe cause of natural bovine mycoplasmal mastitis,[16,17] and as few as 70 colony forming units have induced mastitis in experimentally inoculated cows.[18] Experimental inoculation of M. bovis is reported to induce arthritis in calves,[19-21] and urogenital tract lesions, infertility,

and abortion in cows.[9,22-24] Mycoplasma bovis inoculation endobronchially or intratracheally into gnotobiotic calves has caused macroscopic, subclinical cuffing pneumonia.[20]

Immunologic resistance to disease resulting from experimental challenge exposure with M. bovis has been reported in naturally infected and vaccinated animals. Vaccination with live or killed M bovis partially protects calves from experimental arthritis induced by intravenous injection with live M bovis.[19] Calves vaccinated with formalin inactivated M bovis have fewer organisms isolated from their lungs after experimental challenge exposure than control calves.[25,26] Reduction of herd morbidity in M bovis mastitis outbreaks is reported using formalized preparations from infected milk,[27] and resistance to experimental intramammary challenge exposure has been demonstrated in lactating cows following natural infection and vaccination.[28,29] The immunologic protection provided by vaccination has not been complete, and some animals or quarters remain susceptible to M bovis disease and/or undesirable hypersensitivity reactions. Although numerous studies have demonstrated measurable humoral and cell-mediated responses to vaccination and natural infection,[18-20,25,26,28,30-33] no correlate of protective immunity has been documented; thus, the mechan-

isms involved in immunologic resistance to M bovis disease remain obscure.

Identification and segregation of infected animals remains the only effective means of controlling M bovis disease.[34] Present methods of identifying mycoplasmal isolates from suspect animals are slow, expensive, and dependent upon serological procedures.[35] Several features of mycoplasmas limit the utility of conventional reagents especially with more rapid and sensitive techniques such as enzyme-linked immunosorbent assay (ELISA). Many mycoplasmas are not potent immunogens and do not evoke production of avid and specific antisera.[33,36,37] M bovis, like other mycoplasmas, incorporates components of the growth milieu in its membrane and, when used as antigen for production of specific antisera, elicits reactivity to growth medium components as well as to innate mycoplasma antigens.[38-43] Immunoglobulin and other serum components may also be present on the surface of mycoplasmal cells.[41,44,45] Therefore, some non-specific crossreactivity between mycoplasmal species will be present when conventional reagents are prepared with whole cells due to immunologic recognition of environment antigens.

No antimicrobial chemotherapeutic agent has proven satisfactory in treating M bovis disease.[21,34] The two main preventive strategies, identification/segregation of infected animals and induction of protective immunity

through vaccination, rely on understanding the immunologic responses to M bovis. This study was undertaken to describe immunologic responses to M bovis antigens in vaccinated and naturally infected animals using some traditional methods and some methods not previously available.

The ELISA is a highly sensitive and specific test now being used extensively in immunologic research and diagnosis.[46-48] The assay has been used to detect the presence of antigens and specific antibody in biological samples. ELISA has been used to detect antibodies to several mycoplasmas.[49-52] The ELISA was adapted for detecting M bovis specific IgG in bovine serum, and the assay parameters were described (Part I).[53]

Characterization of M bovis antigens has recieved little study despite the importance of the organism as a bovine pathogen. Preliminary studies were undertaken using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), ELISA, and gel-electrophoresis-derived enzyme-linked immunosorbent assay (GEDELISA) using various antigens and antisera from rabbits hyperimmunized with homologous and heterologous mycoplasmas to identify crossreactive and M. bovis specific antigens (Part II).[38]

The GEDELISA, which combines the high resolving power of SDS-PAGE to separate complex molecules by molecular weight with the sensitivity of ELISA to measure specific antibodies,[54,55] was adapted for detection of bovine serum

antibodies specific for M. bovis antigens. The method was used to assay the antigenic specificity of the serum immune responses from cows resistant and cows susceptible to experimental challenge exposure to live virulent M bovis. If antigens important in resisting disease were elucidated, they would be useful in measuring and inducing protective immunity in vaccinated and naturally infected animals (Part III).[56]

Previous studies have demonstrated tissue tropism for respiratory mycoplasmas,[57-59] and immune responses in vaccinated and naturally infected calves.[14,30,31] A study of tissue tropism and immune responses in natural M. bovis respiratory infection was undertaken using ELISA adapted to describe the serum and secretory isotypic M bovis specfic antibody responses in addition to other measurements of humoral and cell mediated immunity. This description (Part IV) should serve as a baseline for comparing the further experimental and field observations.

Clinical and experimental observations indicate that natural infection or vaccination with M bovis may induce resistance to reinfection.[25-29] Vaccine preparations achieving promising results have used adjuvants,[19,25-27] which may overcome the putative immunosuppressive effects of M bovis.[31] M bovis antigens in combination with adjuvant would be extremely useful if a pronounced local, long lasting immune response could be achieved. Five adju-

vant preparations were combined with M bovis antigens and administered to groups of calves. Cytologic, microbiologic and immunologic measurements were made during vaccination and experimental challenge exposure to determine efficacy of these preparations. (Part V).

Understanding the specific immunologic responses will lead to better control of M bovis bovine disease. This manuscript is submitted as a contribution toward achieving that goal.

PART I: Detection of *Mycoplasma bovis* Specific IgG in Bovine Serum by Enzyme-Linked Immunosorbent Assay

SUMMARY

The enzyme-linked immunosorbent assay (ELISA) was optimized for detection of Mycoplasma bovis-specific IgG in bovine serum. The test is rapid, reproducible, convenient, and sensitive. With this assay, the serum from naturally infected and immunized calves demonstrated the presence of antibodies early in infection and rapid increase in titers during the infection. Cross-reactivity of bovine serum with mycoplasma antigens of bovine, caprine, avian, and environmental sources was tested with this assay system. Cross-reaction was measurable in all instances, with the strongest reaction measured between M bovis and M agalactiae.

INTRODUCTION

In studies on the antibody response of cattle to Mycoplasma bovis, the primary pathogenic mycoplasma causing mastitis and other diseases in dairy cattle, tests such as agglutination, latex agglutination, metabolic inhibition, indirect hemagglutination (IHA), growth precipitation and growth inhibition, double immunodiffusion, and single radial hemolysis were used.[1-15] Although the IHA test is considered sensitive, reliable, and highly specific, low titers have been demonstrated in animals not exposed to M

bovis, and some recently infected animals may not show any titer.[3,7]

Bruggman et al[16] developed an enzyme-linked immunosorbent assay (ELISA) after the method of Engvall and Perlman[17] to detect antibodies to M suipneumoniae in pigs. Specific antibodies were detected 5 weeks before, and 50 weeks after, the clinical manifestation of enzootic pneumonia in specific-pathogen-free pigs.[16] Since the ELISA method has been shown to be extremely sensitive in detecting specific antibodies to a variety of antigens, including mycoplasmas, we adapted the technique for detecting M bovis specific antibodies in sera of calves and cows experimentally and naturally infected with M bovis.[16,18-20]

MATERIALS AND METHODS

Serum Samples--Serum samples for controls and optimization of test procedures were obtained from a cow with a chronic M bovis infection. Negative serum samples for controls and optimization testing were obtained from an animal with no record of having had M bovis infection. The ELISA was performed on sera from 12 calves (14 weeks old) obtained from a commercial dairy calf-rearing facility to measure the increase in specific serum immunoglobulin (Ig) levels in response to vaccination and naturally occurring infection.

Antigen Preparation--Mycoplasma bovis strain California 201 and other mycoplasma antigens were prepared as described elsewhere,[21] and stored in 1-ml amounts (containing 1 mg of protein/ml) as determined by the Bradford method.[22] These were thawed, sonicated briefly,[a] and diluted 1:200 (in 0.1 M Na_2CO_3, 0.02% w/v NaN_3; pH = 9.6) as needed.

Serotest Procedure--Serum was analyzed for M bovis-specific antibodies by IHA, as previously described, and the ELISA.[21] The ELISA for M bovis-specific IgG was conducted following the method of Bruggman et al.[16] Test procedures were optimized as described under Results, using variations of the final procedure adopted as described herein. Briefly, 0.05 ml of antigen was incubated (in duplicate) in wells of Linbro 96-well microtitration multiwell plates[b] for 2 hours at 37 C and washed with 0.15 M NaCl and 0.05% w/v Tween 20.[c] Test serum (0.05 ml, diluted 1:100 in phosphate-buffered saline solution [PBSS] with 0.05% w/v Tween 20) was added, incubated 15 minutes at room temperature in the wells, and washed 4 times with NaCl and Tween 20. Next, conjugate[d] (0.05 ml, diluted 1:1,000 in PBSS with 0.05% w/v Tween 20) was added, incubated 15 minutes at room temperature in the wells, and washed 4 times as before. After adding 0.10 ml of substrate (0.4 mM ABTS,[e] 1.5 mM H_2O_2, 0.05 M citrate; pH = 4), color was developed for 15 minutes at room temperature, and then

0.20 ml of stopping reagent was added (0.1 M hydrofluoric acid, 0.01 M NaOH, 0.001 M EDTA; pH = 3.3), and the absorbance was measured at 415 nm on a Titertek Multiskan.[b] Results were expressed in terms of absorbance at 415 nm or in terms of an ELISA value in which the mean positive reference serum absorbance was set equal to 1, the mean negative reference serum was set equal to zero (0), and the test absorbance was scaled according to this 2-point regression. Four positive and 4 negative control sera were tested on each plate.

RESULTS

Optimization of Antigen--Earlier studies with sonicated, delipidized, and sodium dodecyl sulfate-treated preparations revealed sonication to be the easiest and most reproducible technique for the preparation of antigen (data not shown).

The optimal antigen concentration was determined by incubating dilutions of antigen in the plates and conducting the ELISA as described. Antigen (1.00 mg/ml) was diluted 1:50, 1:100, 1:150, and 1:200 in carbonate buffer before application to the plate. The results are given in Table 1. Antigen was diluted to 1:200 (= 0.25 ng/well) without significant loss of absorbance; therefore, this concentration of antigen was used in subsequent assays.

The optimal incubation time for antigen adsorption to the Microtiter plates was determined for 0.25 ng of sonicated antigen/ml. Plates were prepared as indicated and incubated for 15, 30, 45, 60, 120, and 180 minutes. The ELISA was conducted and the results are given in Figure 1. Incubation at 2 hours was chosen as the optimal time for antigen adsorption.

Optimization of Serum-- Serum was diluted 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1,600 with serum diluent, and the ELISA was performed. The results are shown in Figure 2. A dilution of 1:100 was chosen, because this dilution permitted differentiation of serum from an infected cow from serum of a noninfected cow.

Serum incubation time was optimized by incubating the serum for 4, 6, 8, 10, 15, 20, 30, 60, 90, 120, and 180 minutes and performing the ELISA as indicated. The results are shown in Table 2. The 15-minute time was chosen as convenient, allowing adequate binding of IgG from the test serum to the antigen on the plate.

Optimization of Conjugate--The optimal concentration of each batch of conjugate for use in the ELISA also was determined. The optimal dilution for conjugates used in our laboratory have varied from 1:50 to 1:2,000 (data not shown).

The optimal incubation time for conjugate was determined by incubating the conjugate for 6, 8, 10, 20, 30, 45,

60, 90, 120, and 180 minutes and performing the ELISA. The results are presented in Figure 3. The time of 15 minutes was chosen as a convenient period, after which negative and positive sera demonstrated recognizably different reactions.

Optimization of Substrate--The substrate concentration was used after the recommendation of Saunders.[23] The substrate incubation times were tested by incubating the substrate for 1, 2, 4, 6, 8, 10, 15, 20, 30, 40, and 50 minutes and performing the ELISA. The results are shown in Figure 4. A 15-minute incubation period was selected, because it represented a convenient time which allowed discrimination between positive and negative test results.

Test Precision--To test the precision of the ELISA, we performed a series of replicate positive and negative tests, using identical reagents. The results are shown in Table 3. The mean positive value was 783.3 $\pm$ 48.0. The coefficient of variation (CV) = [(SD/mean absorbance) X 100] for positive serum was 6.1. The mean negative value was 175.8 $\pm$ 48.2 (CV = 27.5).

Sensitivity--The sensitivity of ELISA was tested by performing assays on serum from naturally infected calves and from calves vaccinated with formalin-killed *M bovis* with and without adjuvants (Freund's complete and incomplete). All calves were challenge exposed (intratracheally and intranasally) with live *M bovis*. The results are shown in Figure 5 (the IHA titers are shown for comparison).

Specificity-- The specificity of the ELISA was evaluated by performing assays with serum from a cow chronically infected with M bovis tested with antigens made from various species of mycoplasmas. This serum demonstrated ELISA reactivity with antigens prepared from M bovis, M agalactiae, strain ST 6, M mycoides var mycoides, M bovirhinis, M gallisepticum, and Acholeplasma laidlawii. The results are shown in Table 4. Reactivity above that of serum from a serologically and microbiologically mycoplasma-negative cow was noted in all cases. The greatest ELISA reactivity was noted with M bovis and M agalactiae.

DISCUSSION

The results demonstrate that under optimal conditions, the ELISA is a fast, reproducible, convenient, and sensitive method for detecting M bovis-specific IgG in bovine serum. The test takes less than 2 hours to complete, and as many as 40 duplicate samples can be assayed on a single Microtiter plate, with several plates conveniently run simultaneously. The ELISA compares favorably with radioimmunoassay in terms of sensitivity and lacks the complications of the latter method regarding preparation, handling, storage, and disposal of reagents. Increase in M bovis titers may be demonstrated in 7 days in vaccinated calves and in 14 days in naturally infected calves (Fig 5). Thus, ELISA may

be useful as a mass screening technique for M bovis infection in dairy herds.

The dilution of test sera for use in ELISA may depend on the intended use of the test. The sensitivity and specificity tolerances acceptable for use in mass screening may differ slighly from those acceptable in evaluating hyperimmune sera, and the dilution of test serum may need to be adjusted accordingly. We chose a 1:100 serum dilution for use in ELISA, because it allowed making discrimination between a cow infected with M bovis and a noninfected cow (Fig 2), and demonstrated reactivity comparable with that of IHA in calves with high titers of antibody (Fig 5).

Since ELISA can detect mycoplasma-specific IgM and IgA, as well as IgG (data not shown), in serum and whey, an antibody-response profile of infected animals can be made.[20] Use of such information regarding the participation of the humoral response in resistance to disease will allow making an evaluation of both the immune status of infected animals and the effectiveness of vaccine preparations in eliciting a protective response.

Although ELISA reactivity was noted with serum from a cow chronically infected with M bovis tested with antigens prepared from several mycoplasmas (Table 4), reactivity with M bovis antigens was considerably higher than reactivity with M bovirhinis or strain-ST-6 antigens. The greatest reactivity was noted with M agalactiae and

M bovis. Because of the unlikelihood of a M agalactiae infection in cattle, cross-reactivity between M bovis and M agalactiae should not deter the use of the test. The cross-reactivity between M bovis and other bovine mycoplasma species needs further study.

[a]Biosonic III, Bronwill Scientific, Rochester, NY.

[b]Flow Laboratories,Inc, McLean, Va.

[c]Polyoxyethylene sorbitan monolaurate, Sigma Chemical Co, St Louis, Mo.

[d]Rabbit anti-bovine IgG-horseradish peroxidase, Miles Biochemicals, Elkhart, Ind.

[e]2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate), Sigma Chemical Co, St Louis, Mo.

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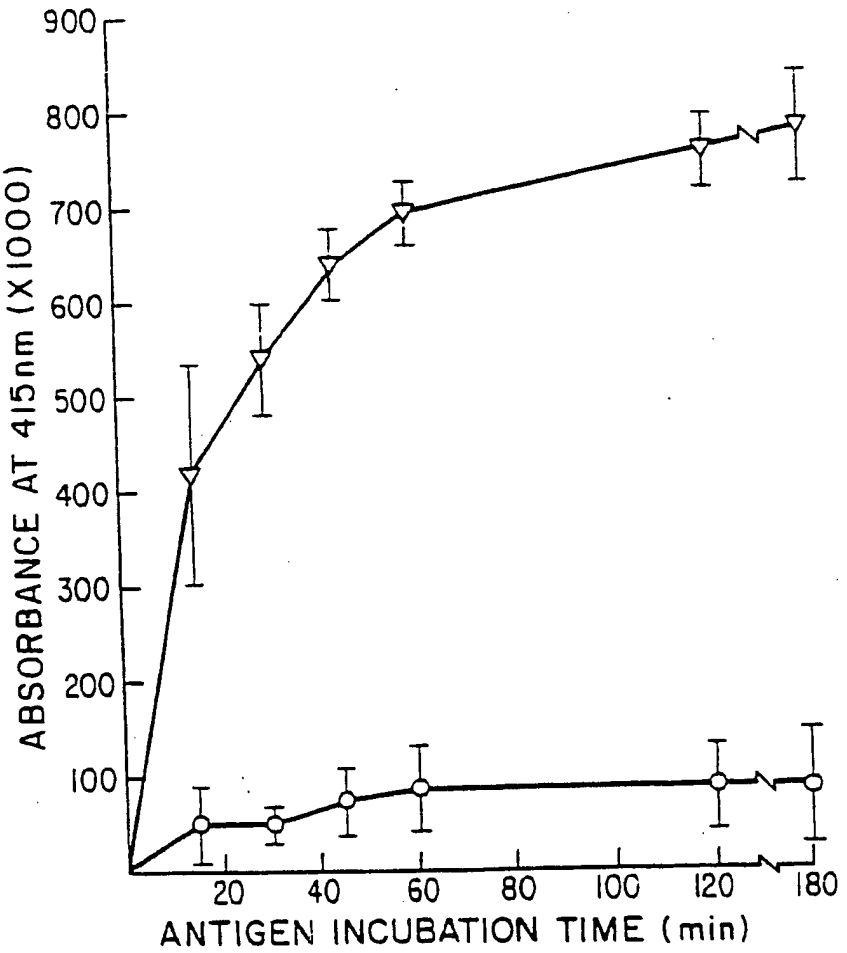
900
800
700
600
500
400
300
200
100
ABSORBANCE AT 415nm (X1000)
20
40
60
80
100
120
180
ANTIGEN INCUBATION TIME (min)

Figure 1. Activity of positive (▽) and negative (○) bovine sera expressed as absorbancy at 415 nm, as measured in the ELISA in which the time of antigen absorption was varied.

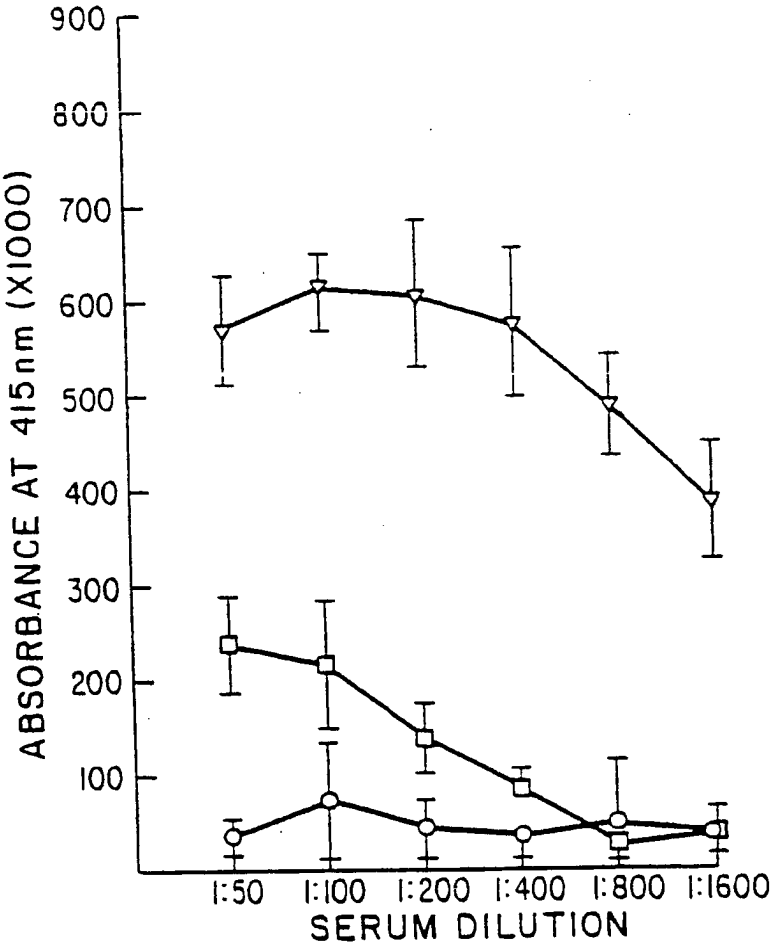
900
800
700
600
500
400
300
200
100
ABSORBANCE AT 415nm (X1000)
1:50
1:100
1:200
1:400
1:800
1:1600
SERUM DILUTION

Figure 2. Activity of sera from a cow chronically infected with M bovis (▽), an animal with M bovis mastitis (□), and a noninfected animal (○) expressed an absorbancy at 415 nm, as measured in the ELISA in which the dilution of serum was varied.

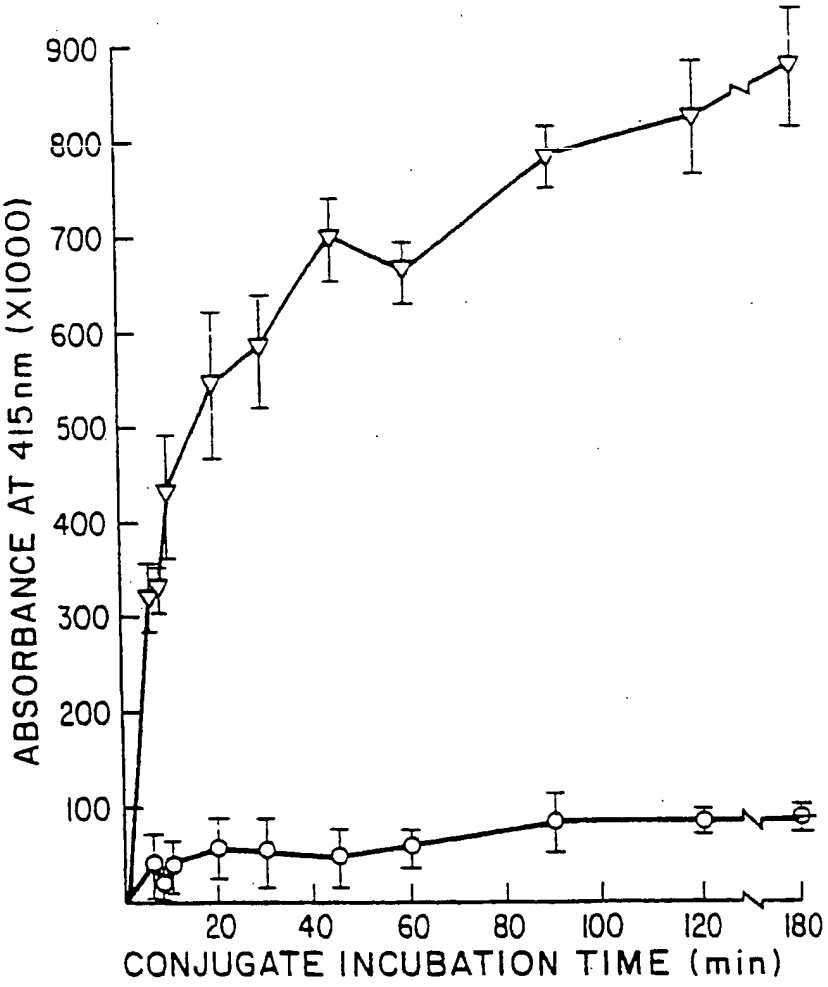
900
800
700
600
500
400
300
200
100
ABSORBANCE AT 415nm (X1000)
20
40
60
80
100
120
180
CONJUGATE INCUBATION TIME (min)

Figure 3. Absorbance readings of positive (▽) and negative (○) bovine sera subjected to ELISA at various conjugate incubation times. Points represent mean absorbance of 8 replicas.

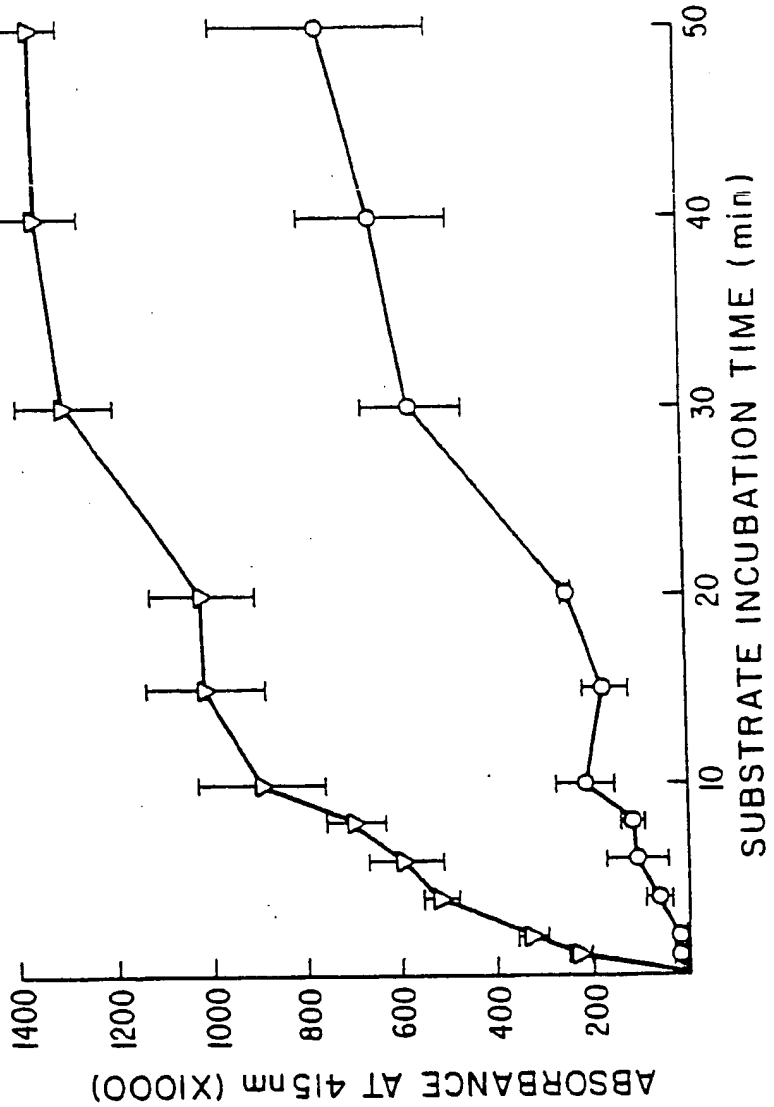
ABSORBANCE AT 415nm (X1000)
1400
1200
1000
800
600
400
200
10
20
30
40
50
SUBSTRATE INCUBATION TIME (min)

Figure 4. Absorbance readings of positive (○) and negative (▽) bovine sera subjected to ELISA at various substrate incubation times. Points represent mean absorbance of 8 replicas.

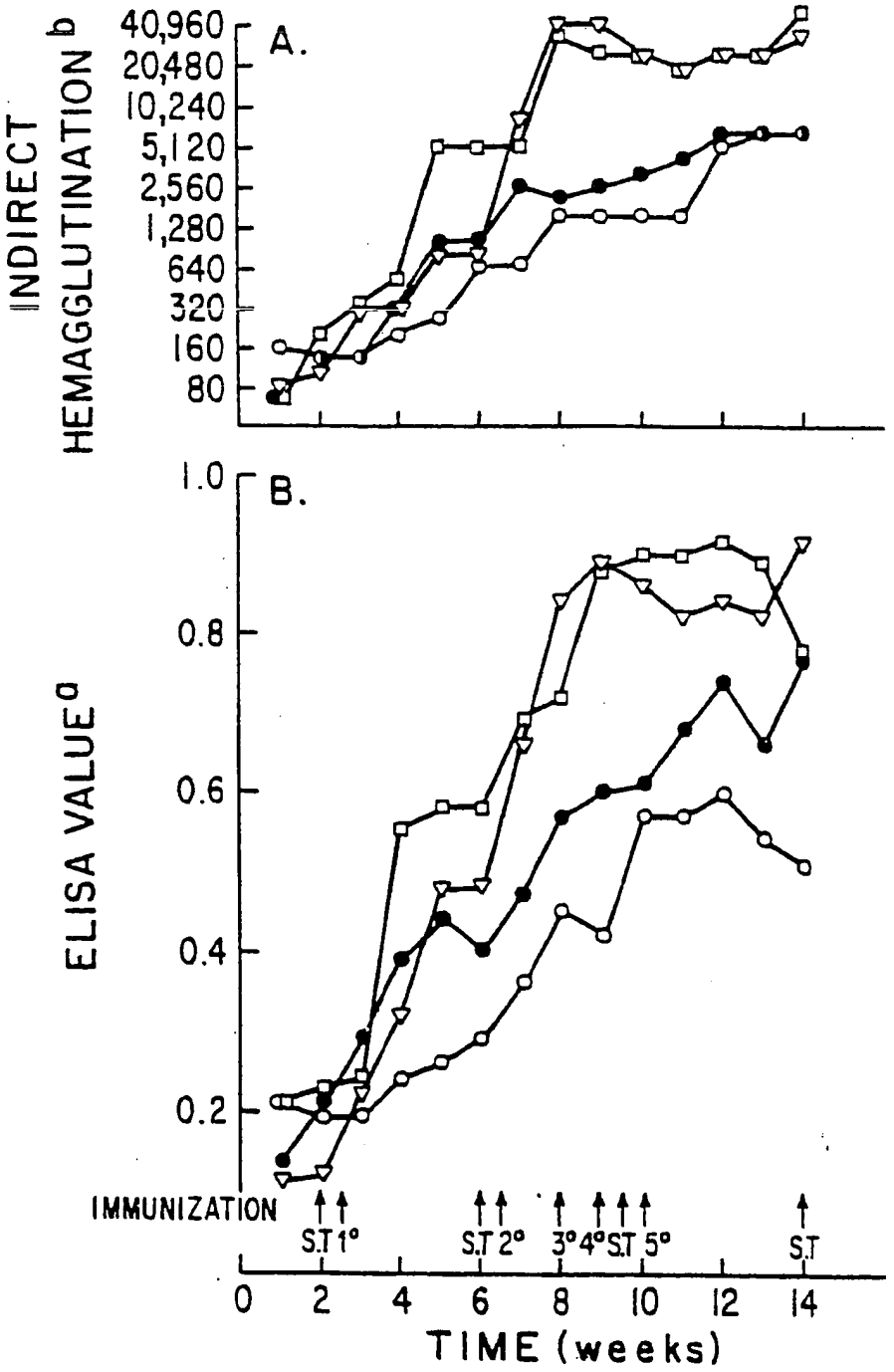
INDIRECT HEMAGGLUTINATION b
40,960
20,480
10,240
5,120
2,560
1,280
640
320
160
80
A.
ELISA VALUE a
1.0
0.8
0.6
0.4
0.2
B.
IMMUNIZATION
S.T 1°
S.T 2°
3°
4°
S.T
5°
S.T
0
2
4
6
8
10
12
14
TIME (weeks)

Figure 5. Measurement of M bovis specific immunoglobulin by IHA (A) and ELISA (B). Points represent mean of duplicate values for 3 calves; naturally infected with M bovis (○), vaccinated with M bovis in saline solution (●), vaccinated with M bovis in Freund's incomplete adjuvant (□), vaccinated with M bovis in Freund's complete adjuvant (▽).
A = ELISA value determined by setting the mean positive reference serum equal to 1, the mean negative reference serum equal to 0, and scaling the test absorbance according to this 2-point regression.
B = Geometric mean titer. Immunization: ST = skin test with 1.0 X 10^9 CFU of formalin-killed M bovis in 0.2 ml at 3 sites in prescapular region, 1° = IM and subcutaneous injection of 500 µg of formalin-killed M bovis, 2° = subcutaneous injection of 500 µg of formalin-killed M bovis, 3° and 4° = intranasal and intratracheal infusion with 1.5 mg of formalin-killed M bovis, and 5° = intratracheal and intranasal infusion with 9.2 X 10^{10} viable M bovis in broth.
Naturally infected calves given 5° immunization only.

Table 1. Absorbance Readings of Positive and Negative Bovine Sera Subjected to ELISA at Various Antigen Concentrations.

	Dilutions* of Antigen†							
	1:200		1:150		1:100		1:50	
	+‡	Neg §	+‡	Neg §	+‡	Neg §	+‡	Neg §
	525	ND	555	174	531	142	552	198
	528	ND	545	166	531	157	560	208
	563	ND	539	186	546	203	576	217
	575	ND	557	192	545	214	571	231
	561	ND	543	162	533	175	589	205
	563	ND	530	196	539	198	589	181
	491	ND	506	154	499	136	538	157
	546	ND	528	229	530	147	801	174
$\bar{X}$	544.0	ND	537.9	182.4	531.8	171.5	597.0	196.4
SD	27	ND	16.5	24.0	14.7	30.4	84.3	24.3

* = Diluted in carbonate buffer. † = Antigen diluted from stock preparation (1.00 mg/ml). ‡ = Positive serum from a cow with chronic M bovis infection. § = Negative sera from animal with no record of having M bovis infection. Neg = negative, ND = not determined, $\bar{X}$ = mean. Values are expressed as absorbance at 415 nm (X 1,000).

Table 2. Absorbance Readings of Positive and Negative Bovine Sera Sujected to ELISA at Various Serum Incubation Times

	Serum incubation time (minutes)											
	4		6		8		10		15		20	
	+*	Neg†	+*	Neg†	+*	Neg†	+*	Neg†	+*	Neg†	+*	Neg†
	243	34	301	38	334	10	343	47	315	37	256	50
	205	41	185	43	246	43	301	45	252	56	324	52
	218	15	208	0	187	4	282	21	254	14	260	9
	264	65	235	72	173	63	297	87	271	73	235	43
$\bar{X}$	232.5	38.8	232.2	38.3	235.0	27.9	305.8	50.0	273.0	45.0	268.8	38.5
SD	26.3	20.7	50.2	29.6	73.2	27.9	26.2	27.4	29.3	25.4	38.4	20.0

	Serum incubation time (minutes)									
	30		60		90		120		180	
	+*	Neg†	+*	Neg†	+*	Neg†	+*	Neg†	+*	Neg†
	350	24	370	12	359	31	394	11	373	3
	344	50	345	54	360	46	306	36	425	50
	338	16	320	25	357	18	342	4	377	45
	352	72	351	76	383	87	341	42	332	58
$\bar{X}$	346.0	40.5	346.5	41.8	364.8	45.5	345.8	23.3	376.8	31
SD	6.3	25.5	18.6	28.8	12.2	29.9	36.3	19.6	38.1	26

* = Positive serum from a cow with chronic *M bovis* infection. † = Negative sera from animal without history of *M bovis* infection. Neg = negative, $\bar{X}$ = mean. Values are expressed as absorbance at 415 nm (X 1,000).

Table 3. Absorbance Readings of Positive and Negative Bovine Sera Sujected to ELISA for Evaluation of Test Precision.

Positive serum*						Negative serum†				
725	748	733	728	758	690	102	163	128	155	193
757	752	775	769	769	738	98	145	127	143	263
800	761	742	751	763	732	111	124	131	142	302
769	781	814	832	786	871	138	181	147	152	ND
777	807	844	752	792	848	182	246	167	154	171
821	803	792	875	775	867	153	187	182	191	287
769	783	829	631	783	814	202	201	124	211	186
813	830	842	812	744	850	207	157	219	209	249
$\bar{X}$		783.3						175.8		
SD		48.0						48.2		
CV		6.1						27.5		

* = Positive sera (1:100) from a cow with chronic M bovis infection. † = Negative sera (1:100) from an animal with no record of having M bovis infection. Values are expressed as absorbance at 415 nm (x 1,000). $\bar{X}$ = mean; ND = not determined.

Table 4. Specificity of M bovis Positive Sera Tested Against Other Mycoplasmatales.

Mycoplasma antigen*	Absorption at 415 nm	
	Positive serum†	Negative serum‡
M bovis	726	126
M bovirhinis	386	103
ST-6	347	110
M agalactiae	705	204
M mycoides var mycoides	202	120
M gallisepticum	298	108
A laidlawii	217	80

* = Antigens (1 mg/ml) Diluted 1:200. † = Positive Serum from a cow with chronic M bovis infection. ‡ = Negative sera from an animal with no record of having M bovis infection. Values are mean of duplicate samples.

PART II: A Characterization of Antigens from Mycoplasmas of Animal Origin

SUMMARY:

Alcholeplasma laidlawii, *Mycoplasma gallisepticum*, *M mycoides* var *mycoides*, *M agalactiae*, *M bovirhinis*, Strain ST-6, and culture medium were compared with *M bovis* by sodium dodecyl sulfate polyacrylamide gel electrophoresis, enzyme-linked immunosorbent assay, and gel electrophoresis-derived enzyme-linked immunosorbent assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated areas of homology and areas of heterology among the species tested. Sera from rabbits hyperimmunized with the mycoplasma and uninoculated culture medium demonstrated enzyme-linked immunosorbent assay reactivity with *M bovis* antigens immobilized on polystyrene. Absorbing the serum from a rabbit hyperimmunized with *M bovis* with culture medium reduced 65.9% of its reactivity with culture medium, 29.7-32.7% of its reactivity with the heterologous species, and 21.1% of its reactivity with the homologous species. Gel electrophoresis-derived enzyme-linked immunosorbent assay performed on immobilized *M bovis* antigens separated by molecular weight using sera from rabbits hyperimmunized with the mycoplasma species under study and uninoculated culture medium revealed antigenic components which are shared among species or with the culture medium, and several which may contain antigens unique to *M bovis*.

INTRODUCTION

Cows infected with M bovis often develop a pronounced immune response.[1] Since pathologic processes often continue in the presence of immune factors which may themselves contribute to tissue damage during infection, it is important to characterize components which participate in immunologic recognition of M bovis.

Immunologic cross-reactivity among members of the Mycoplasmatales is well documented.[2,3] Antigens responsible for such cross-reactivity should be characterized since serologic methods used rountinely for identification of mycoplasmas are compromised by the limited specificity of available reagents.

Cross-reactvity has been detected by immunodiffusion,[4] agglutination,[5] mycoplasmacidal assays,[6] growth precipitation,[7] two dimensional immunoelectrophoresis,[8,9] enzyme-linked immunosorbent assay (ELISA)[8,10,11] and a variety of other methods. Cross-reactive antigens have been associated with culture medium components, with various members of the genus, and with other bacteria and viruses.[5-7,9,12-14]

Reactivity to M bovis, Strain ST-6, M bovirhinis M agalactiae, M mycoides var mycoides, M gallisepticum, and A laidlawii has been demonstrated in serum from a bovine infected with M bovis using ELISA.[10] Recently, gel electrophoresis-derived enzyme-

linked immunosorbent assay (GEDELISA), which combines the high resolving power of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate complex molecules by molecular weight with the highly sensitive ELISA to detect specific antibodies, was used to characterize the immunologic reactivity of M bovis antigens with bovine sera from 8 cows experimentally infected with M bovis.[15]

The present study was initiated to characterize M bovis, Strain ST-6, M bovirhinis, M agalactiae, M mycoides var mycoides, M gallisepticum, A laidlawii, and the medium used for cultivation of the organisms by the following techniques: 1) SDS-PAGE, to visualize protein complexes separated by molecular weight, 2) ELISA, to measure the reactvity of serum from a rabbit hyperimmunized with M bovis against whole mycoplasmal cells and uninoculated culture medium immobilized on polystyrene, and 3) GED-ELISA, to measure the reactivity of sera from rabbits hyperimmunized with whole mycoplasmal cells and uninoculated growth medium against M bovis antigenic components separated by molecular weight and immobilized on polystyrene.

MATERIALS AND METHODS

Preparation of mycoplasmal whole cell antigens--M bovis, Strain ST-6, M bovirhinis, M agalactiae, M mycoides var mycoides (large colony type),[16] and A

laidlawii cultures were prepared from stock organisms maintained in our laboratory. Mycoplasma gallisepticum was provided by H. E. Adler, Department of Epidemiology and Preventive Medicine, School of Veterinary Medicine, University of California, Davis. Mycoplasmal cells used in SDS-PAGE, ELISA, and GEDELISA analysis, and in the preparation of antisera in rabbits, were prepared as described elsewhere.[17] Briefly, a cloned culture of each mycoplasma was used to inoculate 50 ml of biphasic mycoplasma medium which was then incubated for 48 hours at 37 C. The culture was then filtered through sterile gauze into 500 ml of modified Hayflick medium containing 15% horse serum. The organisms were recovered by sedimentation at 27,000 x G and washed 3 times in phosphate buffered sterile saline solution (PBSS) pH 7.2, diluted to 1.00 mg protein/ml as determined by the Method of Bradford,[18] and stored at -20 C in 1 ml aliquots.

Preparation of antisera in rabbits--Antisera against each mycoplasmal whole-cell antigen, formalized by the method of Adler,[19] were raised in rabbits. Antiserum was also raised in a similar manner against uninoculated culture medium. Methods and schedule of inoculation are presented elsewhere.[20] Normal rabbit serum was obtained from Antibodies, Inc., Davis, California.

Adsorption of serum from a rabbit hyperimmunized with M bovis whole cell antigen was performed with medium com-

ponents immobolized covalently on sepharose 4B[a] to remove antibodies reactive with culture medium. Triplicate 60-minute adsorptions were performed by a batch process following the recommendations of the manufacturer.

SDS-PAGE--SDS-PAGE was performed as described by Laemmli using a 5% stacking and 8% running gel.[21] Analytical gels were performed using 1.5 mm thick gel and preparative gels were performed using a 3.0 mm thick gel. All gels were electrophoresed at 10 C until the dye front had migrated 25 cm into the running gel. Protein separation was visualized with Coomassie Brilliant Blue. Molecular weights were calculated from standards[b] lysozyme (14,300), soybean trypsin inhibitor (21,000), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (68,000), and phosphorylase B (94,000).

ELISA--ELISA for detection of antibodies directed against whole mycoplasma cells and growth medium components was performed as described elsewhere.[10] Briefly, 0.05 ml of antigen was incubated in duplicate wells of Linbro 96-well microtiter plates[c] for 2 hours at 37 C and washed with 0.15 M NaCl and 0.05 w/v polyethylene sorbitan monalaurate[d] (Tween 20). Test serum (0.05 ml, diluted 1:200 on phosphate buffered saline solution (PBSS) with 0.05% w/v Tween 20 was added. The plates were incubated for 15 minutes at room temperature, followed by four washes. Next, conjugate[e] (0.05 ml, 1:1,000 in PBSS with 0.05% w/v

Tween 20) was added, incubated 15 minutes at room temperature in the wells, and washed 4 times as before. After adding 0.10 ml substrate [2,2'azino-di-(3-ethylbenzthiazoline) sulfonic acid[d] 0.4 mM, H_2O_2 1.5 mM, Citrate 0.05 M, ph = 4.0], color was developed for 15 minutes at room temperature, 0.20 ml stopping reagent was added [0.1 M hydrofluoric acid, 0.01 M NaOH, 0.001 M EDTA, pH = 3.3], and the absorbance was measured at 415 nm on a spectrophotometer.[f]

Results are expressed in terms of absorbance at 415 nm or in terms of an ELISA value (EV) in which the mean positive reference serum absorbance was set equal to one, the mean negative reference serum was set equal to zero, and the test absorbance was scaled according to this 2 point regression. Four positive and four negative controls were assayed on each plate.

GEDELISA--GEDELISA was performed as described elsewhere.[15] Briefly, 400µg M bovis protein, California Strain 201, was disrupted for 5 minutes at 100 C and applied to a 3 mm x 14 cm x 30 cm slab gel electrophoresis unit.[g] The preparation was electrophoresed through a 3% stacking and 8% running gel at 10 C until the dye front had migrated 25 cm into the running gel. The gel was then recovered and a vertical 15 mm x 25 cm section was cut from the gel and stained for protein with Coomassie Brilliant Blue. The remainder of the gel was cut into 50, sequential horizontal

sections (5 mm each) and transferred to individual 12 mm x 100 mm test tubes. The antigenic complexes were eluted with PBSS with 2.0 mM phenylmethyl sulfonylfluoride[d] and 0.02% sodium azide.[g] Eluate from each tube was immobilized in separate wells of 96-well polystyrene plates and tested for reactivity with various rabbit sera by ELISA.

Analysis--All ELISA values and graphical representation were done with the aid of a computer[h], using programs developed at the University of California, Davis.[i] All statistical analyses were done on the same system using prepared programs.[j]

RESULTS

SDS-PAGE--Results of an analytical SDS-PAGE performed on M agalactiae, Strain St-6, M mycoides var mycoides, M bovis, molecular weight markers, A laidlawii, M gallisepticum and culture medium are presented in Figure 1. No protein band was common to all species tested but areas of homology among species and culture medium can be observed.

ELISA--Reactivity of serum from a rabbit hyperimmunized with M bovis test against whole cell antigen prepared from A laidlawii, M gallisepticum, M mycoides var mycoides, M agalactiae, M bovirhinis Strain ST-6, M bovis, and uninoculated culture medium is shown in Table 1. The greatest reactivity was detected with

M bovirhinis antigen (EV 1.052) followed by M bovis (EV 1.000), A laidlawii (EV 0.990), M agalactiae (EV 0.941), M gallisepticum (EV 0.916), Strain ST-6 (EV 0.895), M mycoides var mycoides(EV 0.641), and culture medium (EV 0.307). When antibodies directed against components of the culture medium were removed by adsorption with medium, the greatest percent decrease in reactivity was noted with medium (65.9%). The reactivity with mycoplasmas from exogenous habitats was reduced 46.5% (M mycoides var mycoides), 40.5% (M gallisepticum), 32.7% (A laidlawii), and 30.9% (M agalactiae). The reactivity with mycoplasmas from the bovine habitat was reduced 29.7% (Strain ST-6), 22.0% (M bovirhinis), and 21.1% (M bovis).

GEDELISA--Protein visualization, fraction numbers, and molecular weight approximations determined for M bovis antigens used in GEDELISA are presented in Figure 2. ELISA reactivity of M bovis fractions separated by molecular weight via SDS-PAGE with antisera raised in rabbits to uninoculated culture medium, A laidlawii, M gallisepticum, M mycoides var mycoides, M agalactiae, M bovirhinis, Strain ST-6, and M bovis, and normal rabbit serum are shown in Figure 3.

Normal rabbit serum demonstrated reactivity with fractions 40 and 49 (Fig 3I) and this reactivity was detectable in all sera assayed (Fig 3A-I). When test serum was omitted

entirely from GEDELISA of M bovis antigens, reactivity at fractions 40 and 49 remained (data not shown).

Serum from rabbits hyperimmunized with M gallisepticum, M mycoides var mycoides and A laidlawii showed reactivity with fractions 36, 40, and 49, and a few other components (Fig 3E-G). Antiserum to A laidlawii also recognized several high molecular weight components in fractions 2-12 (Fig 3G).

Antiserum to M agalactiae demonstrated reactivity with fractions 6, 19, 29, 32-35, and 37-38, as well as with fractions 36, 40, and 49 (Fig 3B). Antiserum to M bovirhinis reacted with fractions 6, 19, 33, 36, 37-38, 40, and 49 in a manner similar to that observed using serum of the rabbit immunized with M agalactiae, but it also reacted with fractions 9, 14, 17-18, 23-25, and 36-42 (Fig 3D). Antiserum to Strain St-6 reacted with fractions 6, 19, 28-29, 32, 36, 38, 40, 49, and a number of high molecular weight components in fractions 1-19 (Fig 3C).

High reactivity of serum from a rabbit immunized with M bovis occurred with fractions 5, 9, 13, 21, 25, 29, 33, 37, 41, 43, and 49 (Fig 3A). This serum reacted with fractions 6, 19, 2, 36, 37, 40, and 49 as did some of the heterologous antisera, but the reactivity with fractions 21, 25, 33, and 43 was relatively unique to the homologous antiserum.

DISCUSSION

Analysis of the mycoplasmas and culture medium presented this study demonstrated the limitataions of SDS-PAGE in comparing relatedness of mycoplasmas using electrophoretic migration patterns. Although migration patterns may indicate characteristic protein profiles useful in species identification, and demonstrate that proteins from different species migrate similarly, statements regarding antigenic relatedness are speculative.[22] Mycoplasma bovis and M bovirhinis, for example, demonstrated a 48,000 molecular weight protein (Fig 1). Serum from a rabbit hyperimmunized with M bovis was highly reactive with an antigen in this molecular weight range as measured by GEDELISA. Serum from a rabbit hyperimmunized with M bovirhinis which contained the 48,000 molecular weight protein (Fig 1) was not reactive with antigens in this range (fractions 28-30, Fig 3D). In addition, not all M bovis antigens demonstrating reactivity with homologous antisera appeared as proteins which could be visualized with Coomassie Brilliant Blue. An M bovis antigen demonstrating reactivity with the homologous antiserum in SDS-PAGE fraction 43 (Fig 3A) by GEDELISA did not stain distinctly with Coomassie Brilliant Blue (Fig 2). Non-protein antigens have been described for a number of mycoplasmas including M bovis.[3,23]

Reactivity of the mycoplasmas considered in this study with serum from a rabbit hyperimmunized with M bovis, as measured by ELISA (Table 1), indicates that much of the reactivity may be due to cross-reactivity with antigens present on other mycoplasmas, and more than 20% of the reactivity may be due to medium components. It is an interesting observation that antiserum raised against M bovis is more reactive with M bovirhinis (EV 1.052) than with M bovis (EV 1.00) in ELISA (Table 1).

The reactivity of antibodies directed to medium components on the mycoplasma cell can be evaluated by comparing ELISA reactivity to each mycoplasma species of serum from a rabbit hyperimmunized with M bovis before and after adsorption with culture medium (table 1). Adsorption removed reactivity with medium (65.9%) to below background levels (EV-0.052), and removed over 30% of the reactivity with mycoplasmas of non-bovine habitats (A laidlawii, M gallisepticum, M mycoides var mycoides, and M agalactiae). Adsorption with medium removed less than 30% of the ELISA reactivity from species of the bovine habitat (M bovirhinis, Strain ST-6, and M bovis) with homologous mycoplasma showing the least loss of reactivity (M bovis 21.1%).

The confounding influence of antibodies directed to antigenic component adsorbable with culture medium obscures the more specific ELISA reactivity between homologous

antigen and antisera. The nonspecific ELISA reactivity observed in ELISA and other serologic tests will be difficult to overcome using antisera raised against conventionally prepared mycoplasma cells grown on complex media. Serologic tests for a variety of pathogenic organisms avoid similar problems by utilizing species- or type-specific reagents.[8,9,13,24,25] The use of reagents made from species-specific antigens which have been demonstrated and characterized for several mycoplasmal species can alleviate this type of nonspecific reactivity.[8,12,24] The GEDELISA technique may prove useful in identifying unique and cross-reactive antigens.

Antigenic components derived from culture media have been detected and characterized for a variety of mycoplasmas including M bovis.[5,7,24,27] The GEDELISA technique using rabbit antiserum raised against culture medium detects antigenic complexes derived from growth medium present in M bovis (Fig 3H). Several slow migrating components (fractions 1-33) and two of lower molecular weight are detectable by GEDELISA in M bovis using this antiserum (fractions 36 and 38). Several of these (e.g. fraction 36) are detectable in M bovis by the sera submitted to GEDELISA, and thus may contribute to cross-reactivity. Two additional fractions (40, 49) demonstrate high ELISA reactivity, but are also reactive with all other sera tested including normal rabbit serum (Fig 3I) and are due to reactivity with

antibodies in the goat anti-rabbit-enzyme conjugate (data not shown).

Several fractions (21, 25, 33, 43) from SDS-solubilized and electrophoresed M bovis antigens demonstratd GEDELISA reactivity almost exclusively with the antiserum raised against M bovis (Fig 3A). These may contain species-specific antigens of M bovis similar to antigens with mycoplasma species-specificity identified by other workers.[12] If further investigation corroborates these findings, improvements in the specificity of serologic reagents, and subsequent serodiagnosis, may be attainable.

[a]Pharmacia Inc, Uppsala, Sweden.

[b]Bio-Rad Laobratories, Richmond, Calif.

[c]Linbro, Flow Laboratories Inc, Hamden, Conn.

[d]Sigma Chemical Co., St Louis, Mo.

[e]Goat anti-rabbit IgG-Peroxidase, Miles Biochemicals, Elkhart, Ind.

[f]Titertek Multiscan, Flow Laboratories, Helsinki, Finland.

[g]Hoefer Scientific Instruments, San Francisco, Calif.

[h]Model 6700, Burroughs Corp, Detroit, Mich.

[i]Developed by Larry Tai, Staff programmer, Division of Statistics, University of California, Davis.

[j]Minitab II Programs, T.A. Ryan, Department of Statistics, Pennsylvania State University, University Park Pa.

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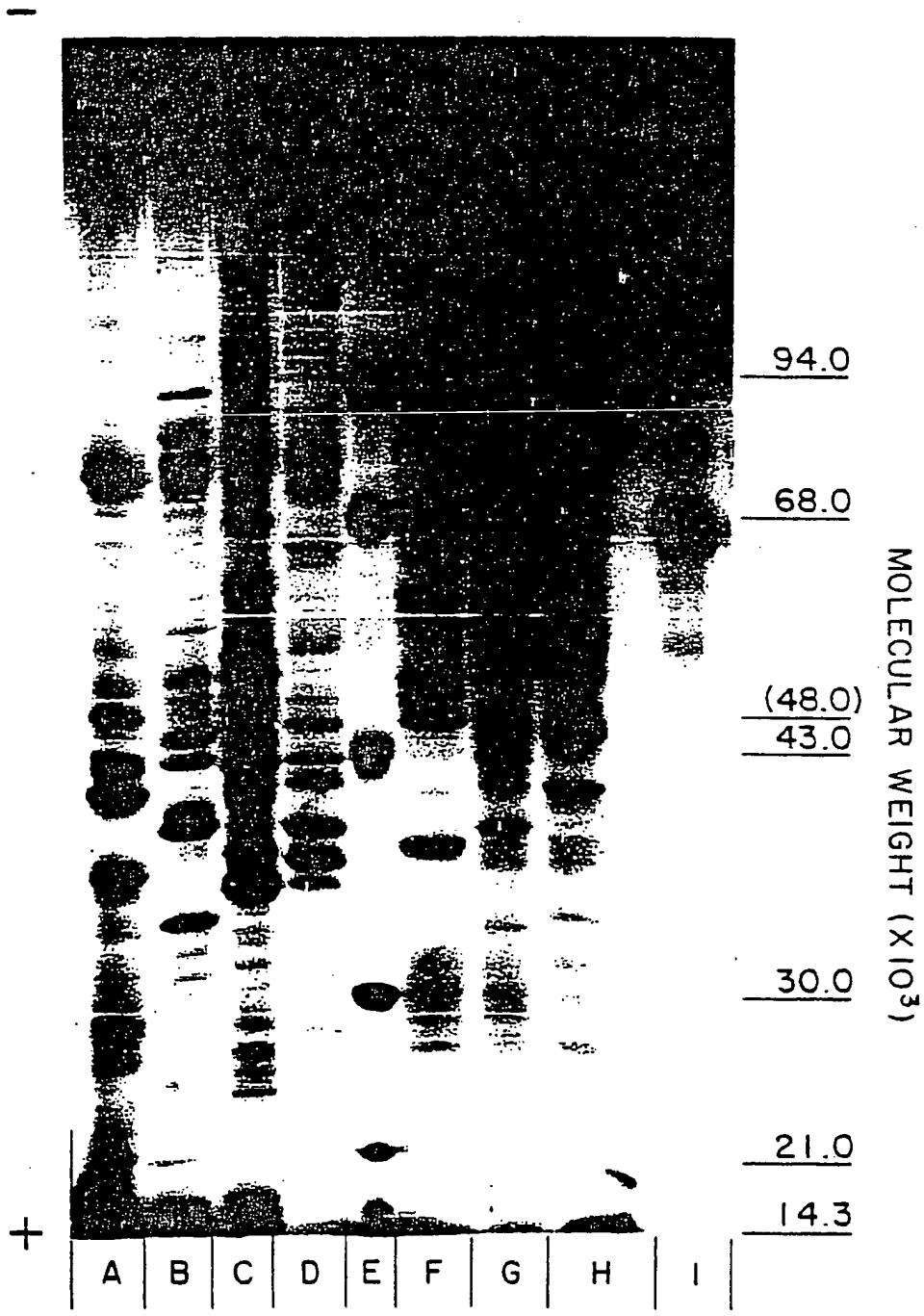
−
+
94.0
68.0
(48.0)
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14.3
A
B
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D
E
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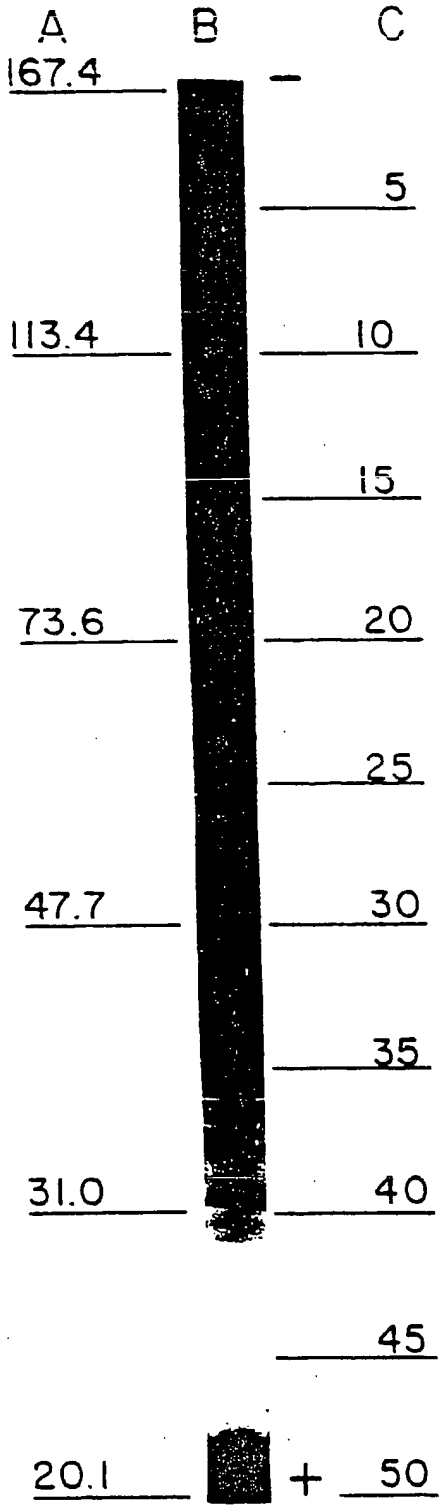
H
I
MOLECULAR WEIGHT (X10³)

Figure 1. Sodium dodecylsulphate polyacrylamide gel electrophoresis of mycoplasmas, culture medium, and molecular weight markers: A) *M agalactiae*, B) Strain ST-6, C) *M mycoides* var *mycoides*, D) *M bovis*, E) Molecular weight markers, F) *M bovirhinis*, G) *A laidlawii*, H) *M gallisepticum*, and I) uninoculated culture medium.


A
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C
167.4
−
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113.4
10
15
73.6
20
25
47.7
30
35
31.0
40
45
20.1
+
50

Figure 2. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of M bovis. A) Molecular weights ($x10^3$), B) SDS-PAGE stained with Coomasie Brilliant Blue, and C) Fraction number used for GEDELISA.

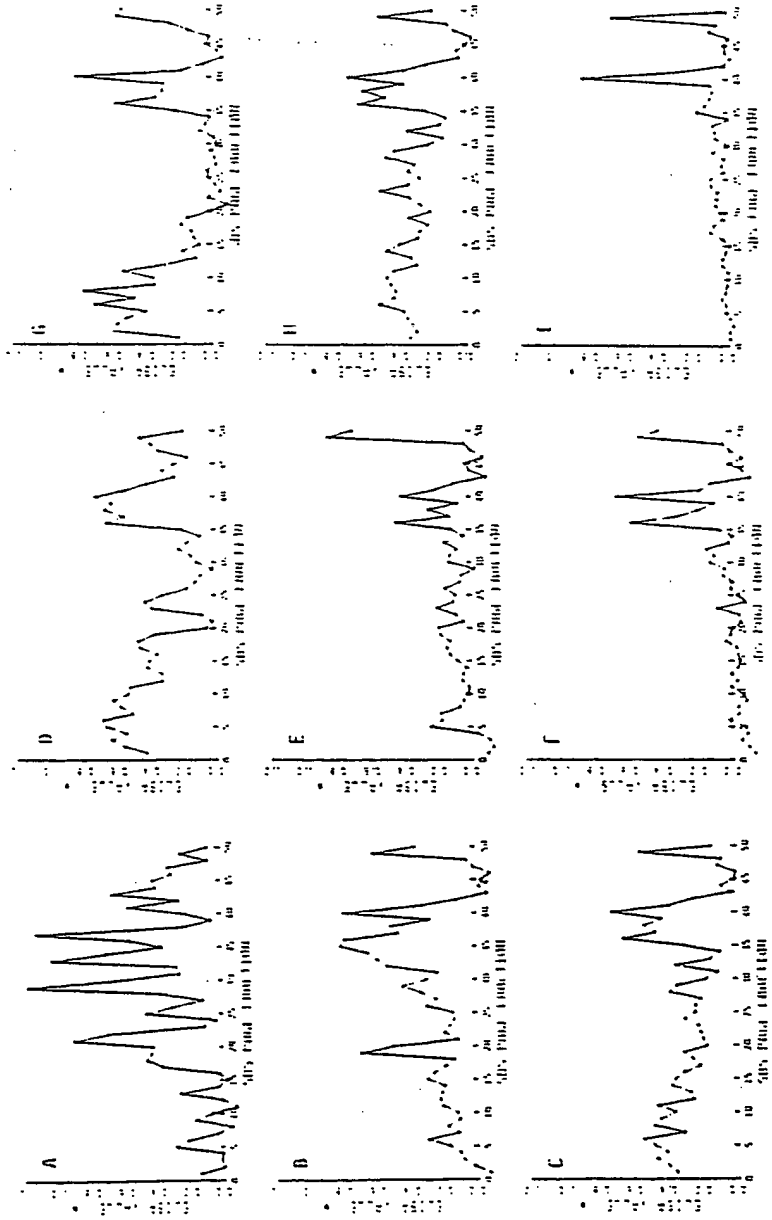

Figure 3. Gel electrophoreis-derived enzyme-linked immunosorbent assay (GEDELISA) results from the sera of nine rabbits tested for reactivity with disrupted M bovis components separated by molecular weight using SDS-PAGE. Profiles were obtained from rabbits hyperimmunized with: A) M bovis, B) M agalactiae, C) Strain ST-6, D) M bovirhinis, E) M mycoides var mycoides, F) M gallisepticum, G) A laidlawii, H) uninoculated culture medium, and I) normal rabbit serum.

*ELISA value determined by setting the mean positive reference serum absorbance equal to one and the mean reference negative serum absorbance equal to zero, and sealing the test absorbance according to this two-point regression.

+SDS-PAGE = sodium dodecylsulphate polyacrylamide gel electrophoresis

Table 1. Reactivity of serum from a rabbit hyperimmunized with M bovis with whole cell antigens prepared from several mycoplasmas and uninoculated culture medium as measured by enzyme-linked immunosorbent assay before and after adsorption with culture medium.

Serum from rabbit hyperimmunized with M bovis[a]		Antigen Immobilized on Polystyrene								
		Culture Medium	A. laidlawii	M. gallisepticum	M. mycoides var mycoides	M. agalactiae	M. bovirhinis	Strain ST-6	M. bovis	M. bovis[b]
Whole Serum	X[c]	427.0	962.0	904.5	688.5	928.8	1010.5	877.8	970.0	186.4
	SD[d]	37.6	20.1	11.6	18.7	17.1	22.6	45.2	36.1	19.7
	EV[e]	.307	.990	.916	.641	.941	1.052	0.895	1.000	0.000
Serum Adsorbed With Medium	X	145.5	647.8	538.0	368.5	641.8	788.3	624.0	765.5	ND[f]
	SD	24.6	21.7	11.5	17.1	27.7	27.7	23.2	38.0	ND
	EV	-.052	.589	.449	.232	.581	.768	.559	.739	ND

Table 1. (con't)

Serum from rabbit hyperimmunized with M bovis[a]	Antigen Immobilized on Polystyrene								
	Culture Medium	A. laidlawii	M. gallisepticum	M. mycoides var mycoides	M. agalactiae	M. bovirhinis	Strain ST-6	M. bovis	M. bovis[b]
Percent of adsorbance removed by adsorption	69.9	32.7	40.5	46.5	30.9	22.0	29.7	21.1	ND

[a]Serum diluted 1:200

[b]ELISA Perfomed on M bovis antigen using normal rabbit serum.

[c]Mean reactivity of quadruplicate test expressed in absorbance (415 nm)

[d]Standard deviation

[e]ELISA value determined by setting the mean positive reference serum absorbance equal to one and the mean reference negative serum absorbance equal to zero, and sealing the test absorbance according to this two-point regression.

[f]Not determined

PART III: Gel Electrophoresis-Derived Enzyme-Linked Immunosorbent Assay on Serum from Cows Resistant to and Cows Susceptible to Challenge Exposure with *Mycoplasma bovis*

SUMMARY

The gel electrophoresis-derived enzyme-linked immunosorbent assay (GED-ELISA) technique combines the high resolving power of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate complex molecules by their molecular weights, with the high sensitivity of the ELISA to detect specific antibody. Sera from 4 cows that demonstrated resistance to challenge exposure and 4 cows that were susceptible to challenge exposure with live virulent Mycoplasma bovis strain 201 were subjected to GED-ELISA to determine reactivity with M bovis antigenic components separated by SDS-PAGE. The GED-ELISA mean reactivity of sera from the 2 groups did not differ significantly (P=0.17) when subjected to analysis of variance. Sera from both groups recognized distinct fractions of M bovis.

INTRODUCTION

Mycoplasma mastitis was first diagnosed in California in 1964[1] and has since been spreading there and in other states.[2,3] Mycoplasma bovis, the most common cause of mycoplasma mastitis,[2] may be an important pathogen in other bovine disease.[4-6] Because treatment has been ineffective in controlling mycoplasma mastitis,[7] recognizing

and eliciting protective immunity are primary research objectives.

Characterization of the humoral serum response has been studied by agglutination,[8] latex agglutination,[8] metabolic inhibition,[9,10] indirect hemagglutination (IHA),[11] growth precipitation and growth inhibition,[12-14] double immunodiffusion,[12,15] and single radial hemolysis.[16] Although IHA has been demonstrated to be a sensitive and reliable test,[7,11] IHA titers do not correlate well with resistance to infection.[17] To date, no test serves as a reliable indicator of current infection or state of resistance or immunity.[2] Recently, an enzyme-linked immunosorbent assay (ELISA) to detect M bovis specific antibodies was developed,[18] but studies correlating ELISA titer to resistance to infection are incomplete.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with its ability to resolve complex proteins by molecular weight, has been combined with several immunochemic techniques, such as crossed immunoelectrophoresis,[19] immunoreplicate electrophoresis,[20] immunofluorescence,[21] and immunoperoxidase,[22] to analyze the antigencity of complex antigens. The SDS-PAGE gives good resolution of mycoplasma organisms and has been used to detect interspecies differences of isolates from several host species.[23-27] The results of 30 mycoplasma isolates

from cattle performed in our laboratory suggested 8 or 9 distinct strains.[28]

Recently, the high resolution of mycoplasma components on SDS-PAGE has been combined with the sensitive ELISA technique to produce the gel electrophoresis-derived (GED) ELISA to characterize M suipneumoniae antigens.[29]

The purpose of the present study was to characterize the serum immunoglobulin (Ig)G response to M bovis by GED-ELISA and to determine whether the technique could detect differences in the immune responses of animals with a history of M bovis infection. Cows that demonstrated resistance to challenge exposure were compared with cows that were susceptible to challenge exposure with M bovis. If the serum IgG response to various antigens differed systematically with resistance and susceptibility, antigens that are important in developing protective humoral immunity could be identified.

MATERIALS AND METHODS

Cows--Eight lactating cows were obtained from various dairies in California and were transported to the experimental animal facility at the University of California, Davis. The cows, experimentally or naturally infected, were challenge exposed 3 times by intramammary injection of viable M bovis strain California 201. Microbiologic culture and serotest results and classification of animals as resis-

tant or susceptible to challenge exposure have been described.[17] Sera from 4 cows (8, 6, 3634, and 20) judged resistant to challenge exposure and 4 cows (809, 948, 4213, and 58) judged susceptible to challenge exposure were submitted to GED-ELISA.

Antigen--The M bovis strain California 201 was prepared as described elsewhere[30] and the protein was determined by the method of Bradford.[31]

GED-ELISA--The SDS-PAGE was performed as described by Laemmli.[32] Briefly, 100 µg of protein of M bovis strain 201 was disrupted for 5 minutes at 100 C and was loaded on a 3-mm X 14-cm X 30-cm vertical slab gel-electrophoresis unit.[a] The M bovis antigen preparation was run through a 5% stacking and 10% running gel at 10 C until the dye front migrated 25 cm into the running gel. The gel was then recovered and a vertical 15-mm X 25-cm section was cut for staining with Coomassie brilliant blue. Molecular weights were approximated by comparing the stained gel with the protein-migration pattern from an SDS-PAGE in which M bovis was electrophoresed in parallel with molecular weight standards[b] [lysozyme (14,300), soybean trypsin inhibitor (21,000), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (68,000) and phosphorylase B (94,000)]. The remainder of the gel was cut into 50 sequential, horizontal sections (5 mm each) and was transferred to individual 13-mm X 100-mm test tubes. The protein was

eluted with phosphate-buffered saline solution (PBSS) with 2 mM phenylmethyl sulfonylfluoride and 0.02% sodium azide.[c]

The ELISA was performed as described elsewhere.[18,33] Briefly, 0.05 ml of each SDS-PAGE fraction was incubated in duplicate wells of 96-well Microtiter plates[d] for 2 hours at 37 C and the plates were washed with 0.15 M NaCl and 0.05% w/v Tween 20.[c] Test serum (0.05 ml, 1:100 in PBSS with 0.05% Tween 20) was added, incubated 15 minutes at room temperature in the wells, and washed 4 times as described. Next, conjugate[e] (0.15 ml, 1:1,000 in PBSS with 0.05% w/v Tween 20) was added, incubated 15 minutes at room temperature in the wells, and washed 4 times as before. After addition of 0.10 ml of substrate [2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid)[c] 0.04 mM, H_2O_2 1.5 mM, citrate 0.05 M, (pH = 4)], color was developed for 15 minutes at room temperature, 0.20 ml of stopping reagent was added [0.1 M hydrofluoric acid, 0.01 M NaOH, 0.001 M EDTA (pH = 3.3)], and the absorbance was measured at 415 nm on a spectrophotometer.[f]

Results are expressed as ELISA value. This was determined by setting the mean positive reference serum absorbance equal to 1, the mean negative reference serum absorbance equal to 0, and scaling the test absorbance according to this 2-point regression. Four positive and 4 negative controls were run on each plate.

Analysis--All ELISA values and graphic representations were done with the aid of a computer,[g] using programs developed at the University of California, Davis.[h] All statistical analyses were done on the same system, using prepared programs.[i]

RESULTS

SDS-PAGE--The SDS-PAGE of M bovis strain 201 stained for protein is shown in Figure 1, with the corresponding fraction number used in the ELISA and approximate molecular weights of immunologically reactive fractions.

GED-ELISA--Figure 2A represents the mean GED-ELISA results from 4 cows judged resistant to experimental challenge exposure with M bovis strain 201. Figure 2B represents the mean GED-ELISA results from 4 cows judged to be susceptible to experimental challenge exposure. An observation of the difference between the 2 groups in mean ELISA value for each SDS-PAGE fraction (data not shown) reveals that fractions 10, 12, 13, 15, 20, 22, 26, 27, and 29 showed differences between resistant and susceptible cows of > 0.05 ELISA value units. Fractions 12 and 27 showed a disparity > 0.10 ELISA value units between groups. The mean GED-ELISA results (Fig 2) demonstrate that fractions 4, 10, 13 to 15, 18, 20, 25 to 27, 31, and 33 contain antigenically reactive components of M bovis separated by SDS-PAGE.

Analysis-- The data were sujected to a 2-way analysis of variance (ANOVA) to examine the significane of differences among the first 35 GED-ELISA fractions (Table 1), and between the 4 cows demonstrating resistance to experimental challenge exposure (20, 8, 6, and 3634) and 4 cows demonstrating susceptibility to experimental challenge exposure (809, 4213, 948, and 58). Variance due to SDS-PAGE fraction was significant ($P < 0.001$), variance due to resistance status of the animals was not significant ($P = 0.17$), and variance due to the interaction of these 2 levels was not significant ($P > 0.25$).

DISCUSSION

The GED-ELISA technique demonstrates recognition of M bovis strain 201 antigenic components separated by SDS-PAGE. At least 3 major antigenic components (fractions 13 to 15, 20, 25 to 27) and 5 minor antigenic components (fractions 4, 10, 18, 31, 33) were recognized in the 8 cows studied in this experiment (Fig 2). The molecular weights of immunologically reactive fractions were approximately 21,000 to 137,000 daltons (Fig 1 and 2).

Genetic composition, state of stress, state of pregnancy and lactation, age, specific immunologic tolerance or suppression, and a myriad of other factors may affect the GED-ELISA pattern in an individual animal. Fingerprinting the immune response by GED-ELISA may be valuable in

characterizing and evaluating the immune response to M bovis under a variety of conditions.

A comparison of cows resistant to experimental challenge exposure with M bovis with those susceptible to experimental challenge exposure by ANOVA showed that the 2 groups did not differ significantly in their mean GED-ELISA values (Table 1). There are several plausible explanations for this observation. Because immunologic differences between susceptible and resistant animals are not known in the case of M bovis infections, determination of adequate sample size (n) is difficult. Mean GED-ELISA differences among groups is relatively small, compared with mean differences among fractions (Fig 2), and larger numbers of animals may have to be assayed to judge the importance of these smaller differences in a more realistic way. The confounding effects of age, genetic composition, state of pregnancy and lactation, and state of stress have not been evaluated and were not studied in this analysis. Individual variation in GED-ELISA responses may obscure real differences between resistant and susceptible animals which may be more subtle.

When examining the differences in antigenic recognition between 2 groups of animals, the state of the antigen used in the assay is critical. Strain 201 used in this GED-ELISA is a laboratory strain passaged many times in artificial medium. Although the resistance status was determined by

experimental challenge exposure with strain 201, resistance to field strains under field conditions may be different. It is not known if immunologically important M bovis antigens are altered during SDS-PAGE, if they are eluted from the gel, or it they stick to the plastic. Thus, GED-ELISA, although sensitive in detecting some antigens, may overlook those important in resistance.

The present study examined the serum IgG GED-ELISA response. Other classes of serum antibody, and specificity and antibody classes produced at local sites are probably more important in resistance to infection by individual lactating quarters than is serum IgG.[2] Although specific antibody must be present for phagocytosis of M bovis by neutrophils and macrophages[34] and increased antibody values are high in protected quarters of lactating cows,[35] the cell-mediated arm of the immune response has been associated with state of resistance[2,7] and may ultimately have a dominant role in protective immunity. In this case, true differences in resistance or susceptibility might not be expressed in the humoral immune response.

The GED-ELISA provides a method for separating and identifying antigenic components of M bovis. Further studies into the mycoplasmacidal abilities of antiserum raised against individual fractions may reveal their immunologic importance. Specific antibody that is increased in protected quarters appears necessary to efficient reticulo-

endothelial function.[34-36] Chemical and biological characterization of antigenic components may reveal their functional role in the mycoplasma cell.

[a]Hoefer Scientific Instruments, San Francisco, Calif.

[b]Bio-Rad Laboratories, Richmond, Calif.

[c]Sigma Chemical Co, St. Louis, Mo.

[d]Linbro, Flow Laboratories Inc, Hamden, Conn.

[e]Rabbit anti-bovine IgG-peroxidase, Miles Biochemicals, Elkhart, Ind.

[f]Titertek Multiscan, Flow Laboratories, Helsinki, Finland.

[g]Model 6700, Burroughs Corp, Detroit, Mich.

[h]Developed by Larry Tai, staff programmer, Division of Statistics, University of California, Davis.

[i]Minitab II Programs, T. A. Ryan, Department of Statistics, Pennsylvania State University, University Park, Pa.

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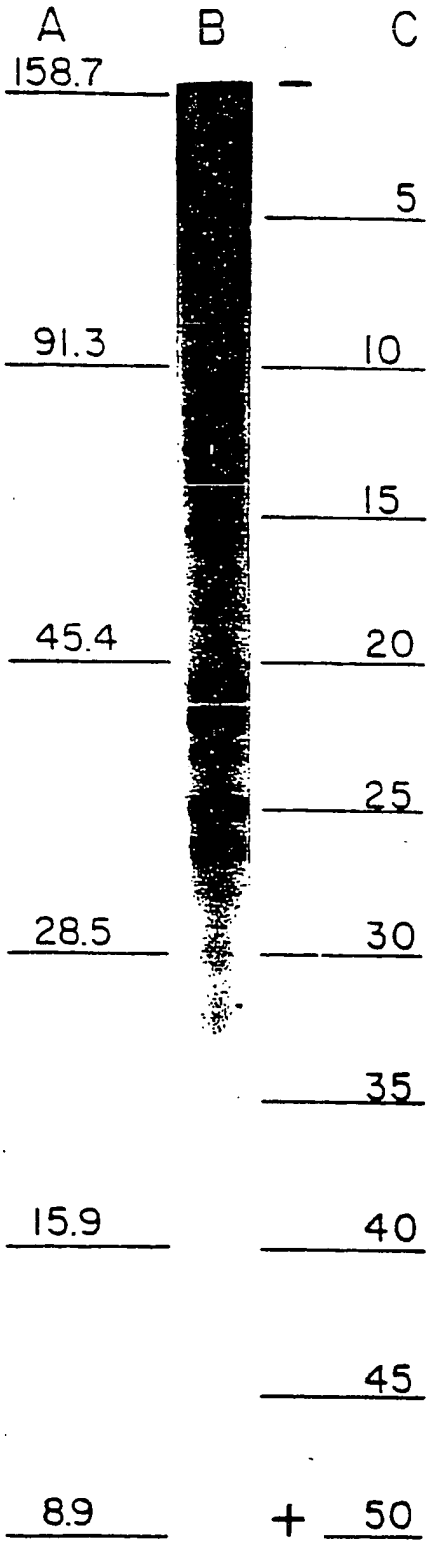
A
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Figure 1A. Approximate molecular weight of GED-ELISA fractions.

B. The SDS-PAGE (10%) of M bovis strain 201 stained with Coomasie brilliant blue.

C. The GED-ELISA fractions.

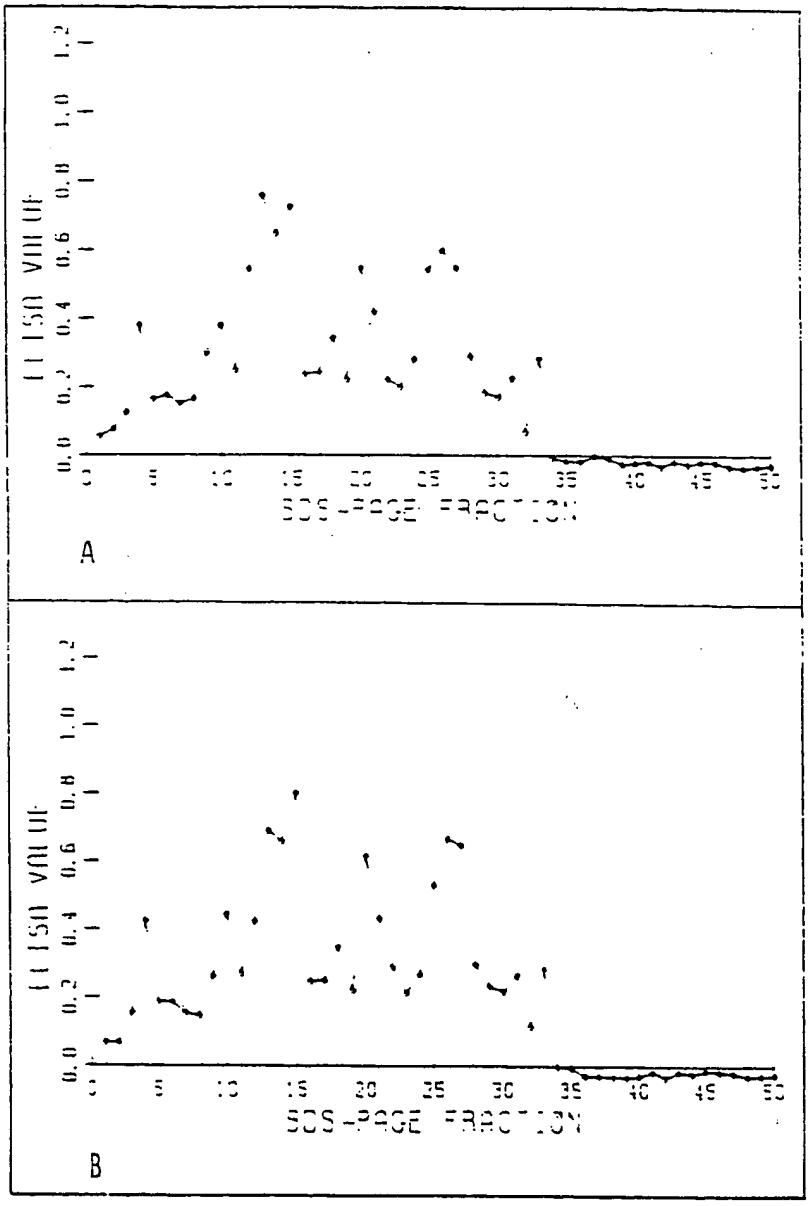
A
B

Figure 2. Composite graphs of the mean GED-ELISA results from cows 6, 8, 20, and 3634 resistant to experimental intramammary challenge exposure with M bovis strain 201 (A) and cows 58, 809, 948, and 4213 susceptible to challenge exposure (B). The SDS-PAGE fraction is plotted against the ELISA value. See text for determination of ELISA values.

Table 1. Two-way ANOVA of ELISA values of resistant and susceptible cows for SDS-PAGE fractions 1 through 35.

Source of Variation	df	Σ of Squares	$\bar{X}$ of Squares	F
SDS-PAGE	34	11.34111	0.33356	38,741
Resistance* status	1	0.01901	0.01901	2.208
Interaction	34	0.11749	0.000346	0.402
Error	210	1.80905	0.00861	---
Total	279	13,28666	---	---

*Cows 6, 8, 20 and 3634 showing resistance to experimental intramammary M bovis infection were compared with cows 809, 58, 948, and 4213 that were susceptible to challenge exposure. See text for determination of ELISA value.

PART IV: Prevalence of Mycoplasmas and Immune Response to *Mycoplasma bovis* in Feedlot Calves

SUMMARY

Microbiologic culture, cytologic, and immunologic observations were made on 30 calves. The eyes, nares, and bronchio-alveolar region were cultured for mycoplasmas. Four of 30 eye cultures, 15 of 30 nasal cultures, and 25 of 30 bronchio-alveolar cultures from the thirty calves were positive for mycoplasmas. Mycoplasma bovis and M bovirhinis were the most prevalent species. Cytology of peripheral blood and bronchio-alveolar washes did not suggest pathologic changes. Indirect hemagglutination, enzyme-linked immunosorbent assay, lymphocyte stimulation tests on peripheral blood cells, and skin testing demonstrated only a low prevalence of immune recognition of M bovis. The natural infection and immune response of 3 calves was followed for 10 weeks before and 4 weeks after intratracheal administration of live M bovis.

INTRODUCTION

Mycoplasmas are responsible for a number of diseases in dairy cattle.[1] M bovis has been isolated from the supramammary lymph nodes, liver, blood, joints, kidney, spleen, lungs and fetus of cows with M bovis infection in the mammary gland.[2] M bovis has been isolated from the respiratory tract of cattle with and without signs of

respiratory disease, and it induces subclinical pneumonia in gnotobiotic calves.[3-10] The respiratory tract of carrier animals may serve as a natural reservoir of M bovis.[11]

Previous infection with some pathogenic mycoplasma in the respiratory tract may confer protection from subsequent infection.[12-17] Experimental systemic and respiratory inoculation with M bovis results in a number of measurable immunologic responses, but immunologic evidence of natural M bovis infection in calves is equivocal.[18-20]

The present study was undertaken to investigate the prevalence of mycoplasma in the eyes, nose, and bronchioalveolar region of healthy calves and to measure the level of local and systemic immune recognition present in calves naturally infected with M bovis.

MATERIALS AND METHODS

Experimental Animals- Thirty Holstein-Friesian steer calves approximately 14 weeks of age were obtained from a commercial calf rearing facility. At the time of selection all calves were culture negative in the nasal passages for M bovis, and had low serum antibody reactivity to M bovis as measured by enzyme-linked immunosorbent assay (ELISA). The calves were transported to the Animal Resource Service facilities at the University of California, Davis, and assigned at random to experimental groups. All calves showed normal temperatures, normal total and differential

white blood cell counts, and good health at all times of observation. They were returned to production at the end of the experiment.

Three calves were selected to be retained for 14 weeks for cultural and immunological observations. Three other calves were selected for vaccination with formalin killed M bovis in Freund's complete adjuvant (FCA). These calves were immunized intramuscularly and subcutaneously (SC) with 500 µg protein from a formalin killed M bovis culture at week 2, SC with 500 µg M bovis protein at week 6, and intranasally (IN) and intratracheally (IT) with 1.5 mg M bovis protein at weeks 8 and 9. The immune status of all 6 animals was challenged by experimental exposure to 9.2×10^{10} c.f.u. M bovis IN and IT at week 10. Cultural and immunologic observations were made on these 3 naturally infected and 3 vaccinated animals for a total of 14 weeks.

Samples--Eyes and nares were swabbed with sterile cotton tipped applicators, incubated overnight in enrichment broth, and cultured on agar for mycoplasma as previously described.[21]

Nasal aspirate for serology was obtained by applying sterile gauze to the nasal passages of the calves. When the gauze was sufficiently moist it was retrieved and the fluid extracted by compressing the gauze in a 6 ml syringe. Approximately 2 ml of sample was obtained. Fifty µl of

thimersol (10 mg/ml) was added to the sample in sterile screw cap vials which were then stored at -20°C.

Bronchio-alveolar region (BAR) lavage samples were obtained through a number 5 French catheter which was introduced into the BAR through a sterile 14 gauge x 2.5" intravenous cannula[a] placed between the tracheal rings using sterile surgical procedure. Twenty ml of physiologically buffered sterile saline (PBSS) was introduced through the catheter using a 30 ml syringe, and the fluid was aspirated immediately. In most cases, 5-10 ml of BAR lavage fluid was recovered for microbiological, immunological, and cytological examination.

Blood was obtained by jugular venipuncture using 10 ml Vacutainer[b] tubes. Samples for serology were collected in Serum Separation[b] tubes. The serum was separated as directed by the manufacturer, placed in screw cap vials, and frozen until use. Samples for total white blood cell count and lymphocyte stimulation test were collected in tubes containing 15.0 mg EDTA.

Microbiology--Bacterial culture was performed on blood agar plates using standard microbiological procedures.[22]

Culture for mycoplasma was performed using modified Hayflick broth medium with 27% (w/v) 1 M K_2HPO_4 buffer and 15% horse serum.[21] Agar medium was prepared by the addition of 1.4% (w/v) agar. Recovered mycoplasmas were

characterized by species using the agar block fluorescent antibody technique.[23]

Cytology--Total white blood cell counts were performed by Coulter count as specified by the manufacturer.[c] Differential white blood cell counts were performed using CAMCOQUICKSTAIN[d] using standard procedures. Cytological analysis of BAR cells were performed on samples layered on glass slides using a cytofuge.[e] The cells were fixed in ethanol, stained with Wright-Leishman stain, and examined by light microscopy.

Serology--Serum was analyzed for M bovis specific antibodies by indirect hemagglutination (IHA) as previously described and by the ELISA.[24,25] The ELISA for M bovis specific IgG was performed as previously described.[26] Results were expressed in terms of ELISA value in which the mean positive reference serum absorbance was set equal to one, the mean negative reference serum absorbance was set equal to zero, and the test absorbance was scaled according to this 2 point regression. Four positive and four negative control replicates were assayed on each plate.

Serum ELISA IgM and IgA values were determined similarly, with the following modifications. Values for M bovis specific IgM were determined using our own conjugate made from guinea pig anti-bovine IgM serum prepared by Dr. E. J. Carroll, Clinical Pathology, Veterinary Medicine, University of California, Davis, using the IgG fraction

purified by ion exchange (DEAE-Sephecel)[f] and conjugated to horseradish peroxidase (Type VI).[g] Values for M bovis specific IgA were determined by applying rabbit anti-bovine IgA (0.05 ml diluted 1:800 in PBSS with 0.05% Tween 20) after washing the test serum from the wells. After a 15 minute room temperature incubation followed by four washes, goat anti-rabbit IgG conjugated to horseradish peroxidase[h] (0.05 ml diluted 1:800 in PBSS with 0.05% Tween 20) was added and allowed to incubate for 15 minutes at room temperature. After washing four times, the color was developed, and the absorbance measured at 415 nm. Data are presented as the M bovis specific IgM and IgA ELISA values as previously described for IgG.

Lymphocyte Stimulation Test (LST)--Blood was collected and the lymphocytes were prepared for culture as described elsewhere.[27] A 0.2 ml aliquot of lymphocyte suspension was applied to wells of sterile 96 well microtiter tissue culture plates[i] and a 0.025 ml aliquot of mitogen (Phytohemagglutin 400 µg/ml, or Concanavilin A 200 µg/ml[g]), antigen (M bovis strain 201, 1 x 10^8 heat killed colony forming units/ml), or medium was added. The cultures were grown for 72 hours at 37° C in a humidified 5% CO_2 atmosphere. Eighteen hours before harvest 0.025 ml of tritium-labeled thymidine (12.5 M Ci/ml)[j] was added. The cells were harvested on a Titertek cell harvester[k] and the counts per minute (CPM) determined by

liquid scintillation.[1] The data are expressed as mean CPM or as the mean stimulation index (S.I. = CPM stimulated culture ÷ CPM control cultures) of triplicate cultures.

Skin Test--Skin sensitivity to M bovis was performed on the lateral aspect of the neck as described elsewhere.[27] Antigen (0.2 ml) was injected intradermally at 3 sites using a 12.5 mm x 25 gauge needle. Reactions were determined with standard vernier calipers by measuring skin-fold thickness in millimeters at 4, 12, 24, 48, and 72 hours. Calves observed for 14 weeks during natural infection and challenge were skin tested with PBSS until week 14 when the skin test was performed as described above.

Statistical Analysis--Stepwise multiple discriminant analysis was performed on the infection status and immune recognition measurements from the 30 calves using the statistical method of Jennrich and Sampson.[28]

RESULTS

Microbiology--A total of 90 initial culture examinations for mycoplasmas were conducted; one for each of the 30 calves from the eyes, nares, and BAR (Table 1). Four of the 30 calves were culture positive in the eyes (M bovis 2, M bovirhinis 1, untyped 1), 15 calves were culture positive in the nares (M bovis 6, M bovirhinis 11, and untyped 1), and 25 calves were culture positive in the BAR (M bovis 14, M bovirhinis 10, untyped 3).

M bovis and M bovirhinis were the predominant mycoplasmas, M bovis being found primarily in the BAR while M bovirhinis was found mostly in the nares. This pattern was confirmed by mycoplasma culture results from the 3 naturally infected calves which were observed for 14 weeks (Table 2). In fifteen BAR, 42 nasal, and 42 eye cultures taken during 14 weeks of observation M bovis predominated in the BAR, while M bovirhinis predominated in the nares. M bovis was present in 12 (80.0%) BAR cultures, 11 (26.2%) nasal cultures, and 2 (4.8%) eye cultures. M bovirhinis was present in 6 (40.0%) BAR, 18 (42.9%) nasal, and 2 (4.8%) eye cultures. Only three mycoplasma cultures from the nares could not be typed as M bovis, M bovirhinis, M canadense or A laidlawii.

Bronchio-Alveolar Region Lavage Cytology--The predominant cells were non-degenerated neutrophils and alveolar macrophages. Some epithelial cells were present. Occasionally, eosinophils, lymphocytes and basophil/mast cells were found. There were no discernible differences in cytology between samples that were positive and those that were negative for mycoplasma or between samples from control and vaccinated calves.

Serum Antibody Response--The initial antibody recognition of M bovis for 30 calves as measured by indirect hemagglutination (IHA) and ELISA, and the culture results for M bovis is summarized in Table 3. The log IHA

titers ranged from 1.602 to 3.107 (mean = 2.023), IgM ELISA values ranged from 0.000 to 0.260 (mean = 0.148), IgG ELISA values ranged from 0.000 to 0.480 (mean = 0.138), and IgA ELISA values ranged from 0.000 to 0.430 (mean = 0.129).

The 3 naturally infected calves and the vaccinated cohort calves demonstrated specific, serum antibody responses as measured by IHA, and ELISA reactivity for IgM and IgG during the 14 weeks of observation (Fig 1A,1B,1D). The serum IgA response as measured by ELISA was low in naturally infected calves even after challenge with live M bovis at week 10 in contrast to the response in vaccinated cohorts (Serum IgA ELISA reactivity is presented in Figure 1D with serum IHA results for clarity of presentation). Serum IHA antibody titers of the 3 naturally infected calves did not rise until the fourth week of observation (Fig 1D). The mean response leveled above 1:1280 at week 8 and produced an anamnestic response to challenge exposure after the 11th week. The response of naturally infected calves never reached the levels of vaccinated cohorts. The serum IHA responses of naturally infected calves and vaccinates were similar until the 7th week, when the vaccinates developed an anamnestic response to secondary systemic administration of antigen. The specific serum IgG reactivity of vaccinates rose markedly above those of naturally infected animals from the 6th week (Fig 1B), and IgA followed suit in the 10th week (Fig 1D).

Secretory Antibody Response--The antibody recognition of M bovis in nasal and BAR lavage fluid sampled at one time for 30 calves as measured by ELISA is summarized in Table 3. In nasal secretions the IgM ELISA values ranged from 0.000 to 0.300 (mean = 0.032), IgG ELISA values ranged from 0.000 to 0.200 (mean = 0.042), and IgA ELISA values ranged from 0.000 to 1.180 (mean = 0.391). In BAR lavage the IgM ELISA values ranged from 0.000 to 0.480 (mean = 0.041), the IgG ELISA values ranged from 0.000 to 0.290 (mean = 0.036), and the IgA ELISA values ranged from 0.000 to 1.030 (mean = 0.230).

The mean IgM, IgG and IgA ELISA values for nasal fluids from the 3 naturally infected calves followed for 14 weeks during infection and their vaccinated cohorts revealed no IgM or IgG response in naturally infected animals (Fig 1A and 1B). The IgA reactivity rose from the first observation and was comparable to vaccinates until the 10th week (Fig 1C). The maximum ELISA IgA reactivity for the natural infection cohort was observed at the fourth week and generally declined after that. Antigenic competition with infecting mycoplasmas, the short half life of IgA at mucosal sites, sampling difficulties, and individuality of calf responses probably contributed to the variability observed in the nasal IgA levels. Serum IgA levels did not rise until the 6th week, whereas nasal IgA levels rose continually during the 1st month in naturally infected calves.

The mean IgM, IgG and IgA ELISA values for BAR lavage from the 3 naturally infected calves and the vaccinated cohort during five periods of the 14 weeks of observation showed a low IgM and IgG response (Fig 1A and 1B). The naturally infected calves showed an early rise in IgA levels which remained elevated but declined gradually following the second observation period (Fig 1C). The vaccinated cohort made no BAR IgA response until the 10th week of observation at which time the full vaccinal protocol had been completed. At week 10 and for the following month the BAR IgA response remained markedly elevated.

Skin Test--The increase in skinfold thickness of the calves (Table 4A) ranged from 0.0 to 14.0 mm (mean = 2.9) at 4 hours, 0.0 to 9.0 mm (mean = 1.4) at 12 hours, 0.0 to 15.0 mm (mean = 1.5) at 24 hours, 0.0 to 5.0 mm (mean = 0.5) at 48 hours, and 0.0 to 0.5 mm (mean = 0.2) at 72 hours.

The mean increase in skinfold thickness of the 3 naturally infected calves and the vaccinated cohort at the beginning and end of 14 weeks of observation (Table 4B) demonstrates that naturally infected animals develop little immediate and no delayed reaction compared to vaccinated animals.

Lymphocyte Stimulation Test--The results of lymphocyte stimulation with M bovis, Con A and PHA are summarized in Table 3. The range of stimulation indices for M bovis was 0.19 to 3.95 (mean = 0.758). The stimulation

indices for PHA ranged from 0.22 to 96.46 (mean = 12.115); for Con-A ranged from 0.64 to 220.58 (mean = 38.105).

The mean initial blastogenic response of the 30 calves (Table 3), and the mean initial and final responses of the 3 naturally infected calves and the vaccinated cohort (Table 5) show that peripheral blood lymphocytes are generally stimulated by Con-A and PHA and suppressed by M bovis compared to control cultures. The stimulation indices indicate that lymphocyte cultures were stimulated less by mitogens and M bovis antigen compared to control cultures after 14 weeks than they were initially. There is considerable variation between animals, and the lymphocyte responses of naturally infected and vaccinated groups are generally similar.

Statistical Analysis--Response variables submitted to stepwise discriminant analysis were IHA, serum IgM, serum IgG, serum IgA, nasal IgM, Nasal IgG, nasal IgA, BAR IgM, BAR IgG, BAR IgA, and the stimulation indices of lymphocytes cultured in the presence of M bovis, PHA, and Con-A. Table 3 shows the mean immune response measurements for 16 calves with positive culture results and 14 calves with negative culture results for M bovis. Using a discrimination level of F. = 4.000, IHA is the only response variable which permits significant discrimination and is highly correlated with specific IgM ELISA reactivity. IHA discriminated between calves culture negative and culture positive

for M bovis in the eyes, nares and BAR with 73.3% accuracy. When the analysis is repeated as above with skin test results from 27 calves (data not shown), IHA remains the only immune response variable which discriminates between the two groups, with an accuracy of 70.4%.

DISCUSSION

Several studies have implicated M bovis as an agent of respiratory disease in calves, and subclinical lesions have been observed in gnotobiotic calves as a result of experimental infection with a number of mycoplasma species.[3,4,8,29] Apart from M mycoides subsp mycoides M bovis is probably the most pathogenic of the bovine mycoplasmas.[30] A number of workers have made concentrated efforts to induce protective immunity to M bovis by various means and determine factors active in resistance.[31-34]

This observational study describes the immunologic responses to natural M bovis infection in which the duration and route of exposure are not known. The fact that all 30 calves were twice originally culturally negative in the nares and did not have elevated levels of M bovis specific serum IgG as measured by ELISA seems to indicate that the infection occurring in the calves was either recent or a condition in which a latent M bovis infection in the respiratory system became more generalized due to the

stress of transport and manipulation. In either case the generalized infection and the immune response seem to be recent events as evidenced by the rise in antibody levels in later observations. Stress has been shown to predispose animals to infecting microorganisms and is strongly implicated in M bovis respiratory disease.[3,4,35]

More than half (16/30) of the respiratory tract samples obtained during the initial observation (Table 1), and those from all 3 naturally infected and vaccinated calves observed for an additional 14 weeks were culture positive for M bovis. During this time, cultures for bacterial pathogens from the same samples were negative, and the calves showed no clinical manifestation of respiratory distress as indicated by behavior, coughing, rectal temperature, or blood and bronchio-alveolar cytology (data not shown). M bovis, although extremely pathogenic in mammary tissue, gave no evidence of clinical pathogenicity in the respiratory tract of these calves. Polymorphonuclear leukocyte infiltration is observed in M bovis infections of the respiratory tract and mammary gland, and was seen in the BAR lavage cytology in this study.[34,36] Eosinophils, sometimes reported in mammary infections, were detected only occasionally in BAR lavage cytology in this study.[34,37]

Since the microbiologic history of these calves is not known they may have been immunized by previous antigenic exposure. This type of protective immunity has been

observed with M pulmonis infections in mice in which initial exposure produced disease, but subsequent infections were asymptomatic.[38] Hypersensitivity, indicated in the pathology of M hyopneumoniae infection in swine and possibly other respiratory mycoplasmas, is probably not a cause of pathology in M bovis respiratory infections since samples from vaccinated calves with high specific serum and secretory antibody levels were obtained from animals with no respiratory disease.[39,40] M bovis may become pathogenic following tissue trauma or other physiological stress. Pathologic synergism between respiratory mycoplasmas and the other respiratory pathogens has been demonstrated experimentally, and coinfection by the bovine respiratory tract mycoplasmas and bacterial pathogens have been observed in cases of feedlot pneumonias.[3,41] Although there is a close association between respiratory disease and M bovis infection, the role of M bovis in respiratory disease is still not clear.

Culture results from nasal swabs and BAR lavage samples indicate that mycoplasmas exhibit tissue tropism within the respiratory tract. A similar pattern was observed by Thomas and Smith in England, where M bovirhinis predominated in the nares of non-pneumonic calves 3-4 months of age.[9] However M dispar predominated in the lower respiratory tract and M bovis was not identified. Observations made

by other workers confirm that respiratory mycoplasmas exhibit tissue tropism.[42]

A study of the nasal prevalence of M bovis in calves from 6 dairy herds by Bennett and Jasper showed that M bovis was present in 6% of animals from herds with no history of mycoplasma mastitis and none of those animals presented respiratory disease.[20] Evidence from the present study indicates that nasal culture probably underestimates the actual respiratory prevalence. Our findings strengthen the hypothesis that the respiratory tract may be a natural reservoir of M bovis organisms capable of causing mastitis.[11,20]

These results emphasize the importance of initial culture and monitoring of respiratory sites, in addition to nasal culture, to determine the state of infection and potential immune response in experiments involving M bovis. The three naturally infected calves observed for 14 weeks developed measurable immune responses despite occasionally negative cultures for M bovis.

These findings present a dilemma to the researcher wishing to study the immune response to vaccination, resistance to disease, and/or pathology of M bovis in the bovine respiratory tract. Procurement and maintenance of immunologically naive field animals may be a prominent obstacle to research. M bovis infection in the respiratory tract or other body sites may be prevalent, and M

bovis can apparently survive for long periods in the environment under ambient conditions.[43,44] Some researchers have resorted to gnotobiotes for experimental work, but translating results to the general bovine population must remain speculative.[29]

The immune responses to M bovis as measured by IHA, ELISA, lymphocyte stimulation and skin test varied greatly among the 30 calves. A discriminant analysis was performed to determine if infected and non-infected animals could be catergorized correctly by measurements of their immune response. If discrimination were possible, measurements of immune recognition might be useful as a screening measure, and immune factors associated with protection from disease might be elucidated. The analysis discriminated correctly much of the time (73.3%) using IHA, and culture negative animals were discriminated with an accuracy of 85.7%. However, only 62.5% of culture positive animals were correctly classified (data not shown). Cho, et al, observed that IHA results provided a better diagnosis of M bovis herd infection than culture isolation and/or growth inhibition in 200 cows from 4 herds.[45] Although discrimination can be achieved using IHA much of the time, use of the assay as a screening measure and the role of agglutinating antibodies and other immune response parameters in M bovis infection needs further study.

The immune response to M bovis infection as measured by IHA, ELISA, lymphocyte stimulation, and skin test on three naturally infected calves was adequate and appropriate to maintain a healthy state during 10 weeks of natural infection, and for 4 weeks after challenge with $>10^9$ CFU of live M bovis. Despite the presumed higher risk of hypersensitivity-related pathologic changes, no evidence of such changes were found in challenged animals having a high immunologic response. There seems to be a wide range of appropriate immune responses.

The serum antibody responses of the 3 naturally infected calves as measured by IHA and ELISA (Fig 1) were in general lower magnitude and less mature (producing less IgG and IgA) at later observations than were those of the vaccinated cohort. The IgM ELISA reactivity of both groups prior to weeks 8 were similar, and paralleled the IHA titers during this period. Bennett and Jasper showed that the M bovis - specific IHA serum titer is largely due to agglutinating IgM in adult cows during the first 50 days of experimental bovine mastitis.[46] The correlation between specific IgM levels and IHA titer is supported by this study since the entrance of IHA into a discriminant function at step 0 (Table 3) drops the IgM F to insignificance to enter step 1.

The IgM and IgG responses were lower, and the IgA responses higher, in nasal and BAR fluids than in serum in

all animals (Fig 1). IgA producing cells stimulated at the local level were probably responsible for comparable serum and mucosal levels of IgA between the two groups until after the 8th week, when vaccinates were given mucosal boosters. The IgA levels of the 3 vaccinated calves in the nares and BAR increased markedly to local stimulation. The importance of local stimulation to evoke a local response is apparent from the response of locally boosted vaccinates, and corroborates the findings of other studies.[32]

The paucity of a secretory IgA response by naturally infected animals to experimental inoculation at week 10 may indicate that M bovis is a common symbiont of the bovine respiratory tract. Such immunologic unresponsiveness in experimental animals to indigenous microflora has been observed previously.[47,48]

Vaccinated calves skin tested at 14 weeks demonstrated both immediate and delayed-type hypersensitivity but little sensitivity was noted in naturally infected calves (Table 4B). The maturation and level of systemic and local antibodies paralleled the skin test responses at 4 and 12 hours. The systemic response of circulating cells at the 14th week of observation is evidenced by the increased skin test responsiveness of the vaccinated calves at 24, 48, and 72 hours compared to naturally infected calves.

Blastogenic responsiveness of circulating cells (as indicated by LST to M bovis antigen) was mostly

suppressed (Tables 3 and 5) compared to control cultures. The responsiveness of lymphocytes from naturally infected and vaccinated calves to stimulation with mitogens and M bovis antigen as indicated by the mean S.I. (Table 5) was suppressed after 14 weeks compared to initial responsiveness. Immune suppression has been observed by others and may be an inherent feature of M bovis infection.[49] The poor correlation between LST and skin test may indicate that LST may not be an accurate correlate of in vivo responsiveness during M bovis infection in the young bovine, or that interference by culture medium components elevate skin test responsiveness in the vaccinated calves.[27,49,50]

A wide range of immune responses were observed in calves with natural M bovis respiratory infections. Among the immune responses submitted to discriminant analysis, IHA best predicted infection status. M bovis demonstrated tropism for the BAR. The humoral immune responses observed in 6 calves during 14 weeks of observation were consistent with those observed with many respiratory infections among the bovine and other animals. LST and skin test results support the contention that naturally infected calves don't develop strong cell-mediated immune responses. Natural M bovis infection and experimental challenge exposure did not produce symptomatic disease among animals with low immune responses, or among animals with

high immune responses which were presumably at risk of strong hypersensitive inflammatory reactions. These observations support the hypothesis that M bovis infecting the respiratory tract of calves may exhibit camouflage and/or immunomodulating capabilities.

[a]Medicut, ALOE Medical Co, St Louis, MO.

[b]Becton, Dickinson and Co, Rutherford, NJ.

[c]Model Z_{B1}, Coulter Electronics Inc, Haialeah, FL.

[d]Cambridge Chemical Products, Inc, Ft. Lauderdale, FL.

[e]Cytospin, Shandon Sourthern Products, Ltd, Runcorn, Cheshire, Eng.

[f]Pharmacia In, Uppsala, Sweden.

[g]Sigma Chemical Co, St Louis, MO.

[h]Miles Laboratory, Inc, Elkhart, Ind.

[i]Bellco Glass Co, Vineland, NJ.

[j]New England Nuclear, Boston, Mass.

[k]Flow Laboratories, Inc, Rockville, MD.

[l]Parkard Tri-Carb Spectrometer, Packard Industries, Downers Grove, Ill.

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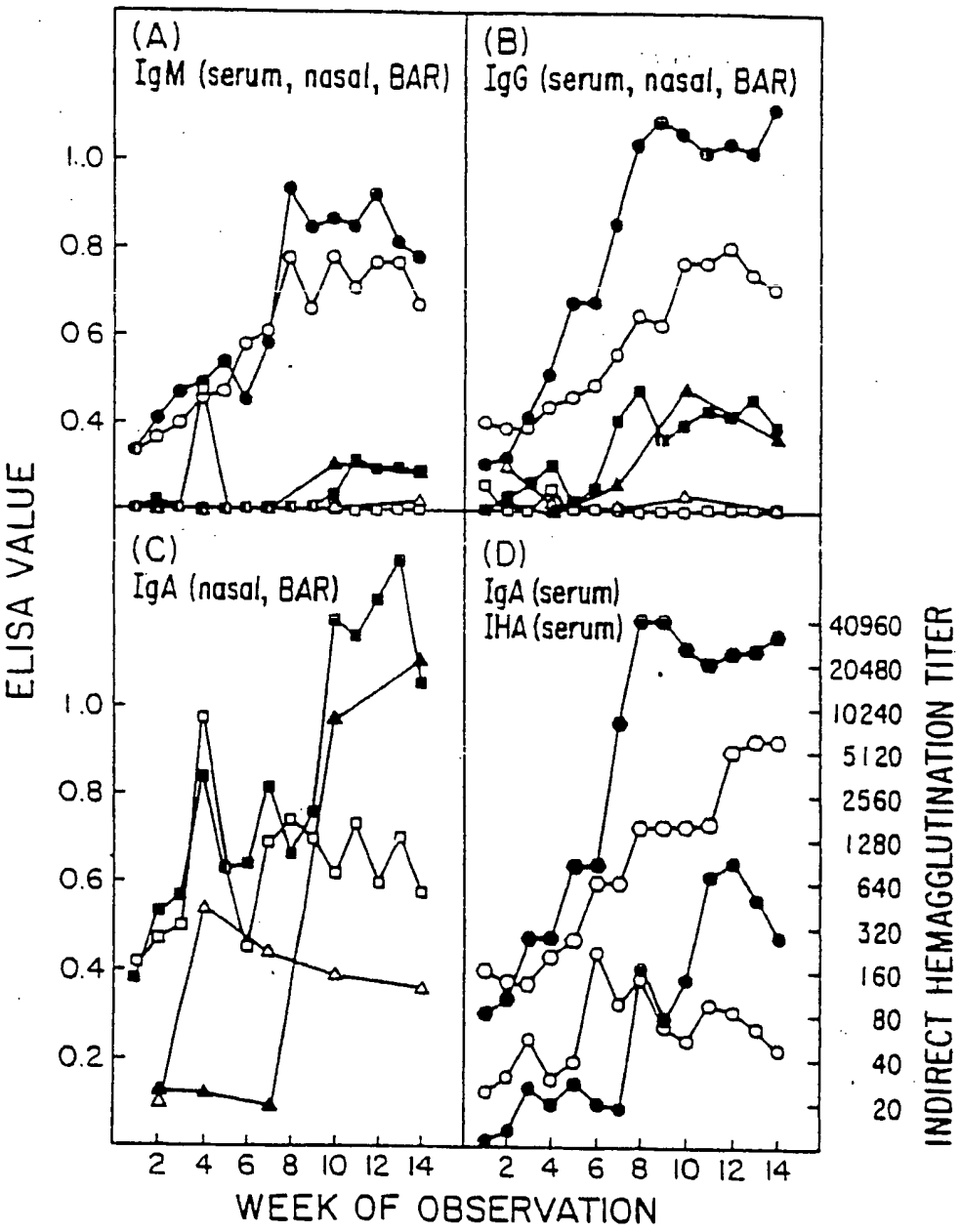
(A)
IgM (serum, nasal, BAR)
(B)
IgG (serum, nasal, BAR)
(C)
IgA (nasal, BAR)
(D)
IgA (serum)
IHA (serum)
ELISA VALUE
1.0
0.8
0.6
0.4
0.2
2 4 6 8 10 12 14
WEEK OF OBSERVATION
40960
20480
10240
5120
2560
1280
640
320
160
80
40
20
INDIRECT HEMAGGLUTINATION TITER

Figure 1. Mean M bovis specific serum indirect hemmagglutination titers (◐) and ELISA reactivity of IgM, IgG, and IgA in the serum (◐), Nasal secretions (◩) and BAR (◭) of 3 calves naturally infected with M bovis and their 3 vaccinated cohorts over a 14 week observation period. (open figures represent values from naturally infected calves; and closed figures represent values from vaccinated cohorts).

*ELISA value see text.

Table 1. Initial frequency and prevalence of mycoplasmas expressed as precent by culture site and mycoplasma species in 30 calves.

Culture Site	Mycoplasma Identification:			
	M bovis	M bovirhinis	Untyped	Totals:
Eyes	2* (6.7%)†	1 (3.3%)	1 (3.3%)	4/30 (13.3%)
Nose	6 (20.0%)	11 (36.7%)	1 (3.3%)	15/30 (50.0%)
BAR	14 (46.7%)	10 (33.3%)	3(10.0%)	25/30 (83.3%)
Totals:‡	16/30 (53.3%)	16/30 (53.3%)	5/30 (16.7%)	

*Frequency of cultures positive for the indicated location and species.

†Prevalence

‡5 cultures from 4 animals contained two species of mycoplasma, and cause the descrepencies in the marginal totals.

BAR = bronchio-alveolar region

Table 2. Results of Mycoplasma culture from the eyes, nares, and bronchio-alveolar region of three naturally infected calves during 14 weeks of observation.

Mycoplasma Identification:	Culture Site:	Calf ID Number:	Culture Results:													
M bovis	Trachea	0	+		−			+			−			+		
		10		+		+			+			+			+	
		20	−				+			+			+			+
	Nares	0	+	−	−	−	−	−	−	−	−	−	−	+	−	−
		10	−	−	+	−	−	+	+	−	−	−	+	+	−	+
		20	−	−	−	+	−	+	−	−	−	−	−	−	−	+
	Eyes	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		10	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		20	−	−	−	−	−	−	−	−	+	+	−	−	−	−
M bovirhinis	Trachea	0	+		−			−			+			+		
		10		+		−			−			−			−	
		20	+				−			−			−			+
	Nares	0	+	+	+	+	−	−	−	−	−	+	−	−	−	+
		10	−	−	−	+	+	+	−	−	−	+	+	−	−	+
		20	−	−	+	−	+	+	−	+	+	+	−	−	−	−
	Eyes	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		10	−	−	−	−	−	−	−	−	−	−	+	+	−	−
		20	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Week of Observation:			1	2	3	4	5	6	7	8	9	10	11	12	13	14

Table 2. (con't)

Mycoplasma Identification:	Culture Site:	Calf ID Number:	Culture Results:													
Untyped	Trachea	0	-		-			-			-			-		
		10		-		-			-			-			-	
		20	-				-			-			-			-
	Nares	0	-	-	+	-	-	-	+	-	-	-	-	-	-	-
		10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		20	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	Eyes	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Week of Observation:			1	2	3	4	5	6	7	8	9	10	11	12	13	14

All calves had negative cultures of the nares at weeks -2 and 0.

- = Negative Culture

Table 3. Stepwise discriminant analysis of immune response measurements from 30 calves cultured for *M bovis*.

Variable*	Mean response from culture negative calves	Mean response from culture positive calves	Mean response from all animals	F to Enter†: Step 0	Step 1
IHA‡	1.796 (0.280)§	2.223 (0.576)	2.023 (0.463)	6.369	----- ¶
Serum IgM¶	0.181 (0.079)	0.118 (0.091)	0.148 (0.086)	4.083	1.447
Serum IgG	0.130 (0.107)	0.146 (0.134)	0.138 (0.122)	0.122	0.000
Serum IgA	0.135 (0.103)	0.124 (0.125)	0.129 (0.115)	0.064	0.003
Nasal IgM	0.026 (0.080)	0.038 (0.078)	0.032 (0.079)	0.166	0.092
Nasal IgG	0.031 (0.053)	0.052 (0.069)	0.042 (0.062)	0.805	0.004
Nasal IgA	0.290 (0.320)	0.479 (0.407)	0.391 (0.369)	1.967	0.514
BAR IgM	0.023 (0.038)	0.056 (0.122)	0.041 (0.093)	0.969	0.023
BAR IgG	0.020 (0.042)	0.049 (0.097)	0.036 (0.077)	1.099	0.158
BAR IgA	0.225 (0.249)	0.235 (0.270)	0.230 (0.221)	0.011	0.049

Table 3. (con't)

Variable*	Mean response from culture negative calves	Mean response from culture positive calves	Mean response from all animals	F to Enter†: Step 0	Step 1
LST‖ - M bovis	0.819 (0.974)	0.704 (0.589)	0.758 (0.791)	0.155	0.202
LST - PHA	9.066 (11.975)	14.782 (23.285)	12.115 (18.896)	0.683	0.259
LST - Con-A	40.966 (64.207)	35.601 (26.587)	38.105 (47.88)	0.094	0.093
Counts:	14	16	30	Total percent classified correctly: 73.3	

*For explanation of variables refer to materials and methods, † stepwise discrimination requires F = 4.000 to enter, ‡IHA = indirect hemagglutination expressed as the mean log titer, §standard deviation, ‖variable removed by stepwise discrimination, ¶antibody levels in serum, nasal secretions, and bronchio-alveolar lavage expressed in terms of ELISA value, # lymphocyte stimulation test (LST) of peripheral blood lymphocytes cultured in the presence of M bovis, Con-A, and PHA expressed in terms of stimulation indices.

Table 4. Increase in skinfold thickness in A) 27 calves at an initial observation, and B) 3 calves which were naturally infected and 3 calves which were vaccinated with *M bovis* after 14 weeks of observation.

Mean increase in skinfold thickness from:		Hours after intradermal injection with *M bovis*: 4	12	24	48	72
A.						
Culture negative calves: (n = 13)	$\bar{X}$	1.2	0.8	0.7	0.7	0.0
	SD	2.1	1.7	1.7	2.5	---
	range	0.0-9.0	0.0-6.0	0.0-5.0	0.0-9.0	---
Culture positive calves: (n = 14)	$\bar{X}$	4.5	1.9	2.3	0.4	0.4
	SD	4.9	3.2	4.1	1.3	1.3
	range	0.0-14.0	0.0-9.0	0.0-15.0	0.0-5.0	0.0-5.0
All Calves: (n = 27)	$\bar{X}$	2.9	1.4	1.5	0.5	0.2
	SD	3.8	2.6	3.2	2.0	1.0
	range	0.0-14.0	0.0-9.0	0.0-15.0	0.0-5.0	0.0-5.0

Table 4. (con't)

Mean increase in skinfold thickness from:		Hours after intradermal injection with M bovis: 4	12	24	48	72
B.						
Naturally Infected calves:						
initial:	$\bar{X}$	nd	nd	nd	nd	nd
	SD	---	---	---	---	---
	range	---	---	---	---	---
after 14 weeks:	$\bar{X}$	7.2	0.0	2.8	2.7	0.0
	SD	7.9	---	4.8	4.7	---
	range	0.0-15.7	---	0.0-8.3	0.0-8.2	---
Vaccinated calves:						
initial:	$\bar{X}$	1.7	2.0	0.7	0.0	0.0
	SD	2.9	3.5	1.2	---	---
	range	0.0-5.0	0.0-6.0	0.0-2.0	---	---
after 14 weeks:	$\bar{X}$	34.6	44.9	34.3	22.7	20.5
	SD	14.9	2.7	5.0	12.7	2.4
	range	18.7-48.3	42.3-47.7	29.7-39.7	10.0-35.3	17.7-22.0

*Measurements in millimeters

Table 5. Mean *in vitro* stimulation of peripheral blood lymphocytes from 3 calves which were naturally infectd and 3 calves which were vaccinated with *M bovis* cultured in the presence of mitogens or mycoplasma antigen.

		PHA	Con-A	M. bovis	Control
Naturally infected calves:					
Initial:	CPM	17530	8246	845	957
	SI	36.37	35.57	1.23	
After 14 weeks:	CPM	20316	22116	859	1131
	SI	19.52	21.71	0.87	
Vaccinated calves:					
Initial:	CPM	18434	63517	2100	3251
	SI	22.97	74.13	0.89	
After 14 weeks:	CPM	37445	32246	2115	1394
	SI	18.28	23.41	0.66	

PHA = phytohemagglutinin, Con-A = concanvalin A, CPM = counts per minute, SI = mean stimulation index (calculated from stimulation indices of individual calves)

PART V: The Effects of Adjuvants on the Immunologic Response of Calves to Vaccination with *Mycoplasma bovis*

SUMMARY

Five groups of 3 calves each were vaccinated with Mycoplasma bovis antigen in phosphate buffered saline solution or one of four adjuvant preparations, and then challenge exposed to live organisms. The adjuvant preparations elicited strong systemic antibody responses compared to those measured in calves not given adjuvant. Most calves given adjuvants responded to vaccination in the respiratory tract with a strong IgA response, but these responses were not above those of calves given M bovis antigen without adjuvant. Calves given some adjuvants developed strong delayed-type hypersensitivity responses compared to calves given M bovis without adjuvant as measured by skin test, but none of the calves developed a strong cellular response as measured by lymphocyte stimulation.

INTRODUCTION

Mycoplasma bovis can be pathogenic in the respiratory tract of gnotobiotic calves and is strongly implicated in outbreaks of pneumonia in feedlot calves.[1-3] The pathogenic factors of respiratory mycoplasmas and the protective role of the host immune system in preventing

respiratory disease due to M bovis infection remains obscure despite extensive studies.[4-10]

M bovis is not highly immunogenic in the bovine, possibly because of similarities between the membrane composition of the mycoplasma and that of host cells and/or the association of environmental antigens with the mycoplasma membrane.[5,11,12] Immunosuppression of humoral and cell mediated responses has been found in calves inoculated with M bovis.[5]

The immunopotentiating effect of adjuvants may be due to prolonged deposition of antigen, modification of the antigen, or the recruitment and/or activation of circulating lymphoid or reticuloendothelial cells.[13,14] Adjuvants have had an enhancing effect on the immunogenic qualities of M bovis antigens, and have been used in successful vaccine preparations of M bovis and other pathogenic mycoplasmas.[5-8,15-18] Adjuvants would be of particular benefit if found efficacious for local immunization where lymphocytes and phagocytic cells are suppressed or where small amounts of antigen is preferred to avoid undesirable reactions.

Observations are presented here on the effect of systemic and respiratory administration of killed M bovis antigen in combination with various adjuvants on the immune responses of calves to vaccination, and to experimental chal-

lenge exposure with live M bovis in the nares and bronchio-alveolar region (BAR).

MATERIALS AND METHODS

Experimental animals--Holstein Fresian steer calves approximately 14 weeks of age were obtained from a commercial calf rearing facility. At the time of calf selection cultures taken from the nasal passages were negative for M bovis, and there was no detectable M bovis-specific serum IgG as measured by ELISA. The calves were transported to the Animal Resource Facilities at the University of California, Davis, and assigned to experimental groups. Three animals were vaccinated with killed M bovis in phosphate buffered saline solution (PBSS), and the remaining 12 were vaccinated with the same antigen in combination with one of 4 adjuvant preparations (3 calves per experimental group).

Evaluation of cytologic, microbiologic, and immunologic data from the calves at this initial time is presented elsewhere.[18] M bovis was identified in mycoplasma cultures from all but two calves during the period from initial culture to the time of experimental challenge exposure infection. Mycoplasma bovis was cultured from the eye, nose, or trachea of all animals after experimental infection. All animals maintained a good state of health for the duration of the experiment.

Mycoplasma strain--M bovis strain California 201 was used for antigen preparation and has been referred to previously with regard to its relationship with other strains.[19]

Vaccine preparations--M bovis antigen was prepared as described elsewhere except that horse serum in the growth was replaced by bovine serum, killed with formalin, and standardized by protein content.[19-21] Antigen was used for skin testing (0.25 mg/ml) and for immunization (5.00 mg/ml).

Preparations for systemic immunization consisted of 0.5 ml antigen, 1.0 ml Freunds Incomplete Adjuvant[a] and 0.5 ml of one of the following in aqueous solution: 10.0 mg N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP),[b] 5.0 mg Amphotericin B (Am-B),[c] 500 mg of combined magnesium/aluminum hydroxide[d] with 2×10^{10} killed Bordetella pertussis[e] (Bp). The other experimental groups received the same antigen in Freunds incomplete adjuvant alone (FIA) or in PBSS without adjuvant. Vaccine preparations for intranasal and intratracheal immunization were the same as those used for systemic immunization except that the oil phase (FIA) and magnesium/aluminum hydroxide were omitted, and the total volume was adjusted to 15 mls with PBSS.

Vaccination schedule--The calves were observed for a period of two weeks before systemic immunizations at week 3 (intramuscular and subcutaneous) and week 6 (subcutaneous).

Intranasal and intratracheal immunizations were performed at weeks 8 and 9. The calves were skin tested with M bovis antigen at weeks 3, 6, 10 and 14. All the calves were challenged exposed at week 10 with 9.2×10^{10} cfu of live M bovis via intranasal and intratracheal infusion.

Samples--Weekly samples were obtained from the 15 calves for immunologic evaluation over a 14 week period. Nasal aspirate, BAR lavage fluid, and blood samples were obtained as described elsewhere.[18]

Serology--Serum was analyzed for M bovis specific antibodies by indirect hemagglutinations (IHA) and ELISA as described elsewhere.[18] ELISA data are expressed in terms of ELISA value (EV) in which the mean positive reference serum absorbance was set equal to 1, the mean negative reference serum absorbance was set equal to 0 and the mean test absorbance was scaled according to this 2-point regression.

Skin Test--Skin sensitivity testing was perfomed on the lateral aspect of the neck as described elsewhere.[6,18] M bovis antigen (0.20 ml) was injected intradermally at 3 sites using a 25 gauge needle. Reactions were determined with standard vernier calipers by measuring skin-fold thickness in millimeters at 4, 12, 24, 28 and 72 hours.

Lymphocyte Stimulation Test (LST)--Blood was collected and the lymphocytes were isolated, quantitated, cultured in the presence of phytohemagglutinin (PHA),

conconavalin A (Con-A), M bovis, or medium alone, pulsed with tritiated thymidine,[f] and harvested as previously described.[5,18] LST data are expressed in terms of counts per minute (CPM) or stimulation indices (SI = Mean CPM of stimulated cultures ÷ Mean CPM of control cultures).

RESULTS

Serum antibody--The calves vaccinated with M bovis antigen is PBSS elicited a moderate IHA response to sytemic vaccination (Fig 1), an IgM ElISA response which declined shortly after the end of systemic vaccination, and a IgG ELISA response which rose througout the vaccination period showing primary (weeks 1-6) and anamnestic (7-14) responses. The ELISA IgA response occurred late in vaccination (weeks 6-9).

The humoral immune responses of calves vaccinated with M bovis antigen in FIA, MDP, BP and AmB (Fig 2-5) as indicated by IHA titer, and IgM and IgG ELISA reactivity occurred faster, reached higher maximum values, and had higher final values than the group receiving antigen without adjuvant. No group receiving adjuvant demonstrated a systematically higher serum antibody response than the group receiving FIA. In general, the ELISA reactivities of serum immunoglobulins indicate that the IgM and IgG responses were higher and the IgA response lower in serum than in respiratory secretions.

Respiratory antibody response--Groups of calves which received M bovis antigen in adjuvants in general responded with some ELISA IgM and IgG reactivity in nasal and BAR secretions above that of the group vaccinated without adjuvant, although the IgM and IgG reactivities were in most cases far below those of serum (Fig 1-5). The IgA response in respiratory secretions was higher than in serum in all but one case (BAR ELISA IgA reactivity of the group receiving M bovis with Bp). All groups receiving adjuvant (except the above group) demonstrated strong nasal IgA responses to respiratory vaccination at weeks 8 and 9. A strong response was also seen in BAR lavage samples to respiratory vaccination (weeks 8 and 9) and experimental challenge exposure at week 10.

The highest respiratory IgA reactivity was found in the nasal secretions of the group which was vaccinated with M bovis in PBSS (without adjuvant). The IgA reactivity in BAR secretions rose after the first observation and was maintained at high levels throughout the study, perhaps due to natural infection.

Skin test: At week 14 all groups of calves had developed skin test reactivity compared to their initial reactivity as measured by increase in skinfold thickness (Fig 6). Among the responses of groups given adjuvant, Bp elicited the lowest mean reactivity at all times measured. Groups given FIA, MDP, and AmB elicited strong responses at

all times measured at 14 weeks. The group given antigen in PBSS elicited low reactivity at 4, 12 and 24 hours, and negligable reactivity at 48 and 72 hours.

Lymphocyte stimulation--Higher mean stimulation indices were measured at 14 weeks compared with initial lymphocyte cultures in all calf groups when cultures were stimulated with PHA, and in all groups except those receiving Bp and AmB adjuvants when cultures were stimulated with Con-A (Table 1). The greatest increases in mitogen and antigen stimulation were measured in the groups receiving FIA and MDP, and the lowest increases were measured in the group receiving no adjuvant.

Lymphocyte cultures from all groups at 14 weeks were suppressed in the presence of M bovis antigens although the suppressive effect had decreased in all cases except cultures from the group receiving Bp adjuvant. When the mean stimulation indices of cultures for all groups is considered, there was an overall increase in mitogen and antigen responsiveness.

DISCUSSION

The role of measurable immune responses in protecting calves from respiratory disease resulting from M bovis infection is not certain. M bovis infected calves with and without a measurable immune response show no respiratory disease, and yet M bovis can be pathogenic.[1,18,22]

Since none of the calves in the present study showed any signs of respiratory illness although a wide range of immune responses were observed, there must be a wide range of acceptable responses to M bovis infection as previously observed.[18]

The use of adjuvants to enhance the immune response may aid in resistance to M bovis disease where immunocompetence is compromised or where immunization with large amounts of antigen to elicit a strong local response causes undesirable reactions. None of the adjuvant preparations used in this study elicited a systemic response much above that elicited using FIA. These responses were comparable to those found using FCA.[18] None of the adjuvants elicited a respiratory antibody response consistently higher or longer than that elicited with local administration of antigen in PBSS, although all adjuvant groups responded strongly to local vaccination. Systemic and local vaccination with killed M bovis resulted in a larger systemic and respiratory response than found in unvaccinated, naturally infected calves observed over the same period.[18]

All groups receiving adjuvant preparations developed delayed-type hypersensitivity at 14 weeks as determined by increase in skinfold thickness at 72 hours. Increased mitogen-induced blastogenesis of lymphocyte cultures occurred in all cases except Con-A stimulated cultures from the Bp and AmB groups. Coincidentally Bp and AmB elicited

lower skin test reactivity than did the other adjuvants. Although LST responses were highly variable between animals, suppression of lymphocyte cultures was a fairly consistant feature corroborating earlier observations.[5,18]

Since no adjuvant preparation used in study produced consistently higher immune responses to M bovis than those produced using FIA, additional modification of M bovis antigens or recruitment and/or activation of lymphoid cells may not be operative.

[a]Difco Laboratory, Detroit, MI.

[b]Syntex Corp., Palo Alto, CA.

[c]E.R. Squibb & Sons Inc., Princeton, NJ.

[d]Moalox, William H., Rores Inc., Fort Washington, PA.

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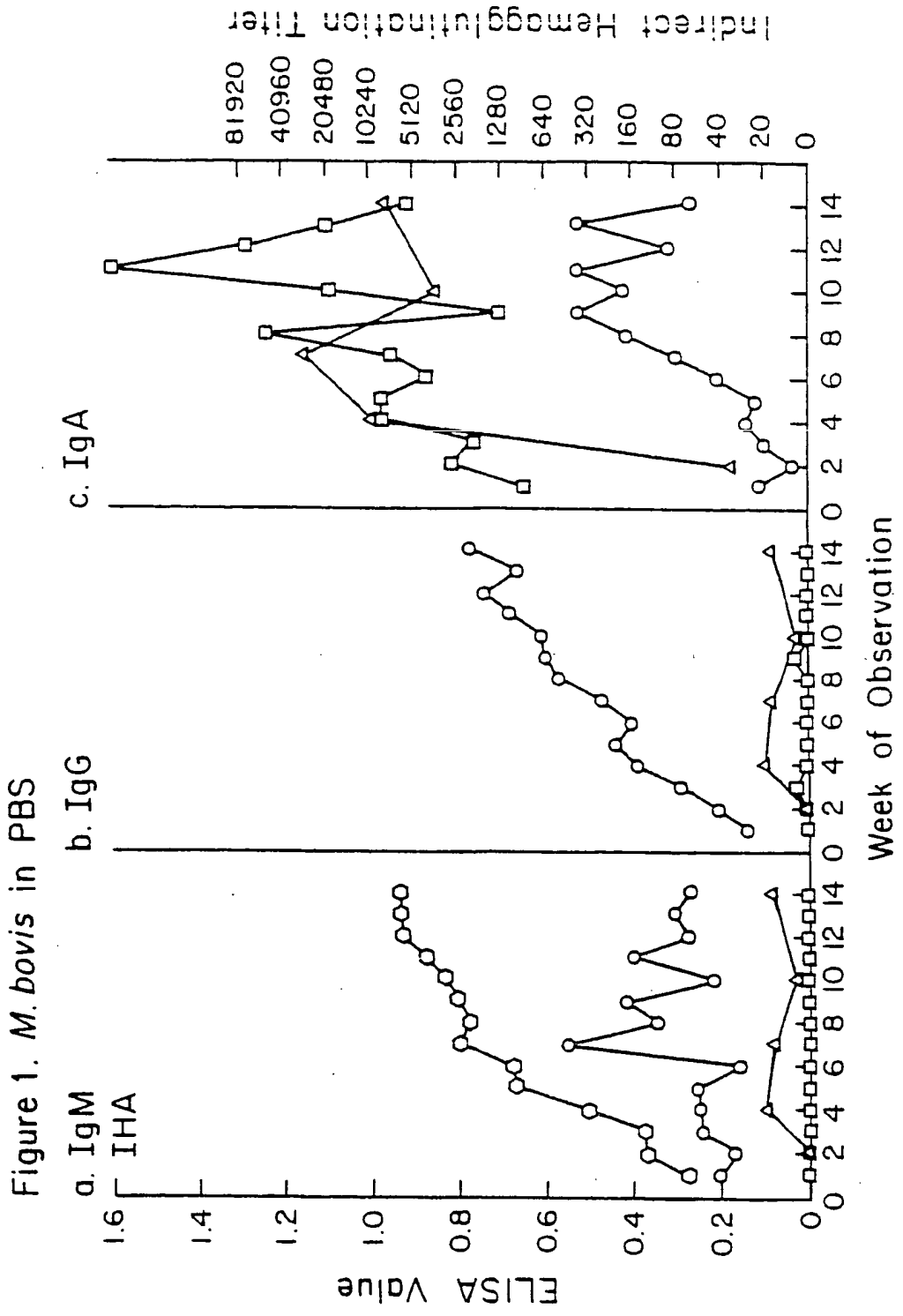

Figure 1. *M. bovis* in PBS

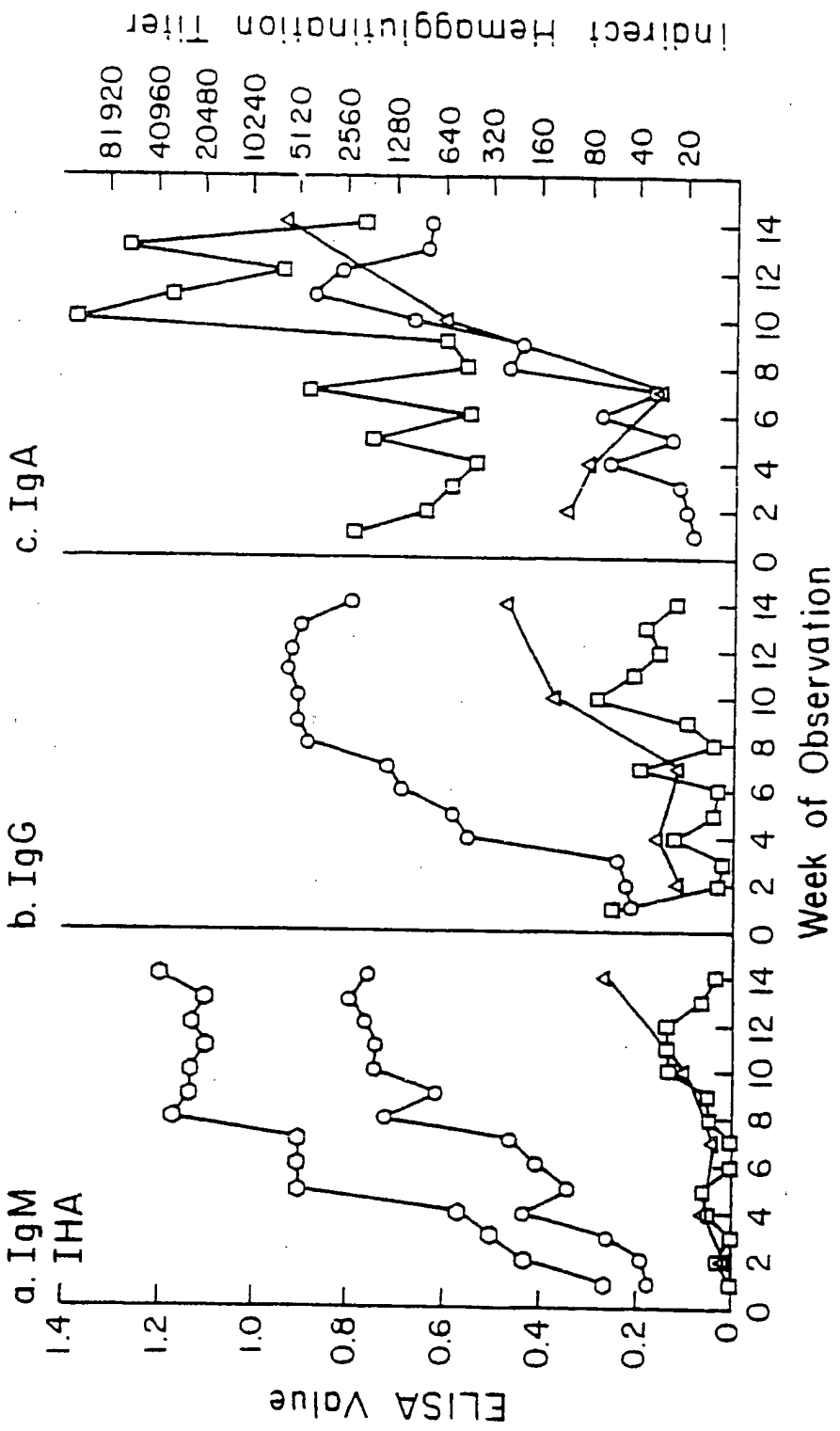

Figure 2. *M. bovis* in FIA

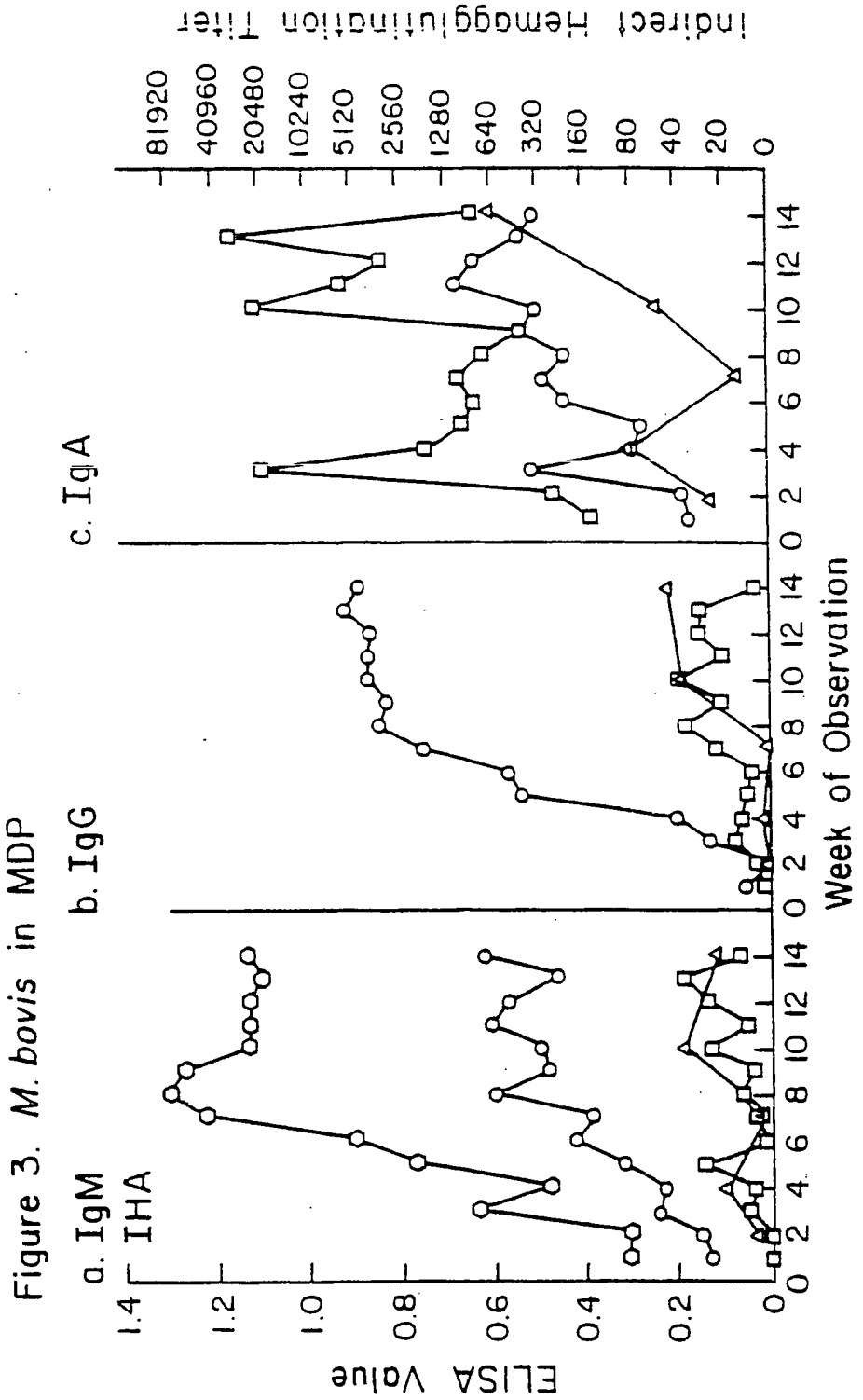

Figure 3. *M. bovis* in MDP

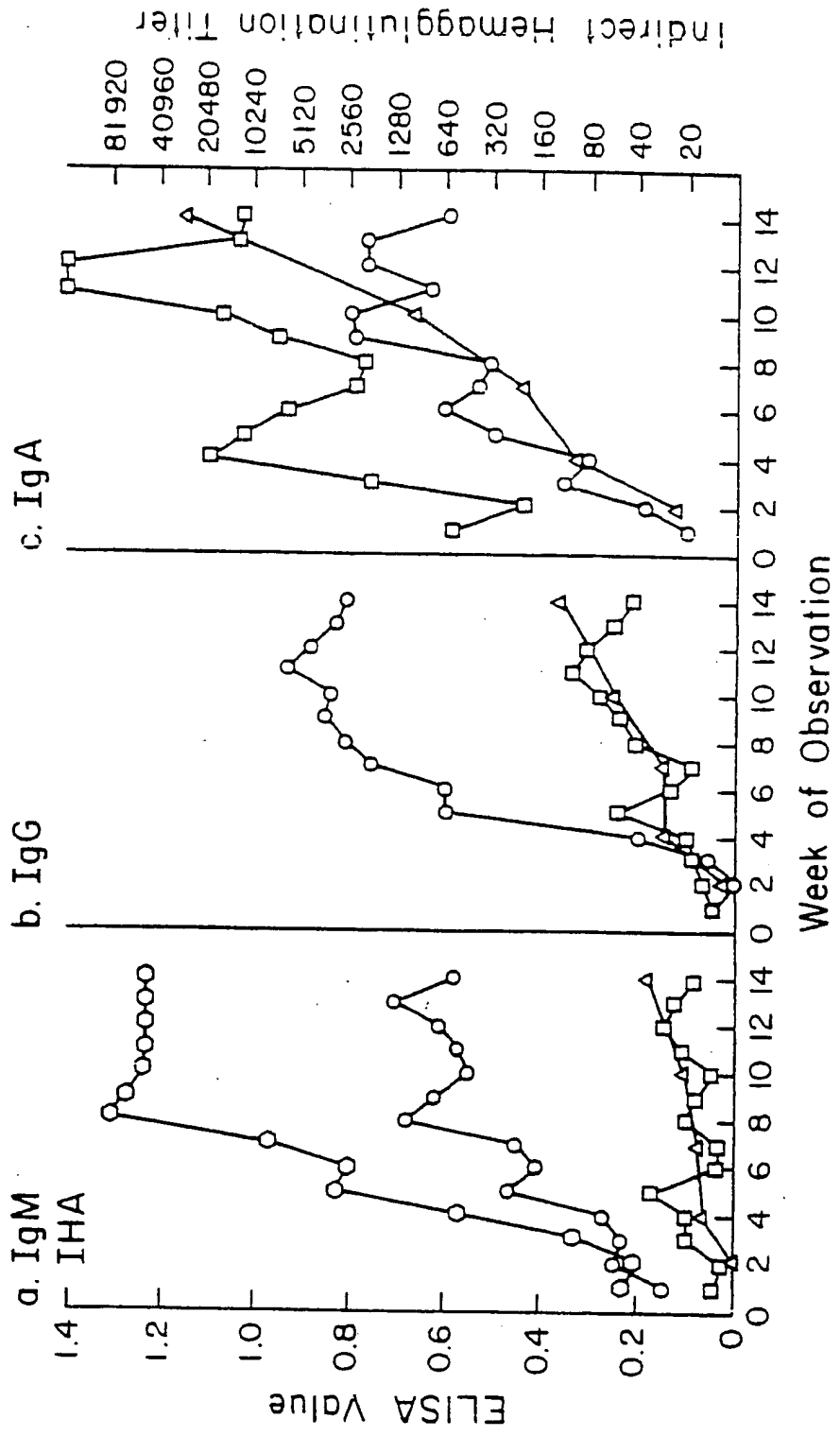

Figure 4. *M. bovis* in AmB

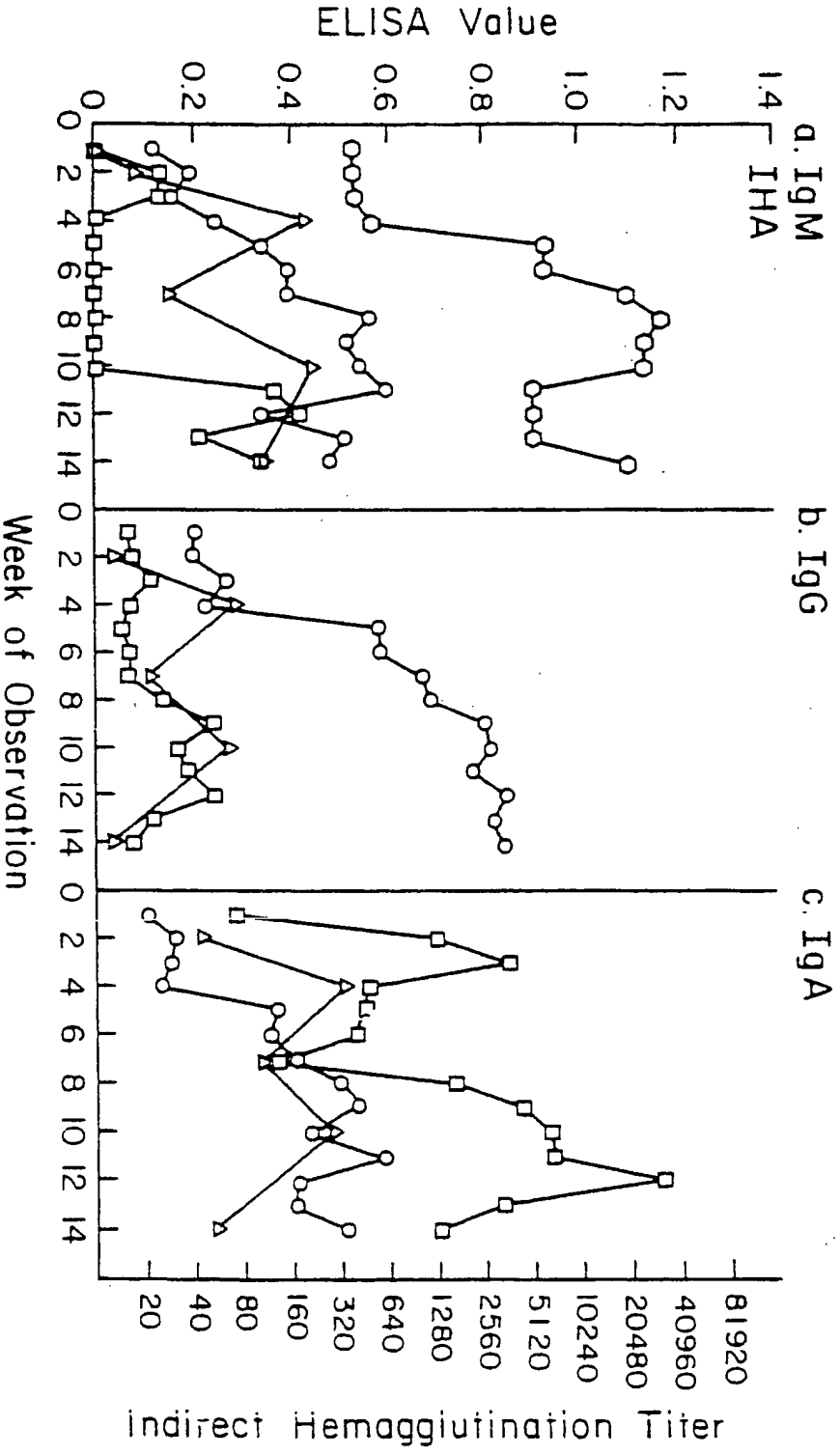

Figure 5. *M. bovis* in Bp

Figures 1-5. Mean M bovis specific indirect hemagglutination titer (Fig 1, ○), and ELISA reactivity of IgM (a), IgG (b), and IgA (c) in serum (○), nasal secretions (□) and BAR lavage (△) samples from calves vaccinated with M bovis antigen in PBSS (Fig. 1), FIA alone (Fig 2), and FIA with MDA (Fig 3), Am-B (Fig 4), or Bp (Fig 5); and challenged exposed to live organisms.

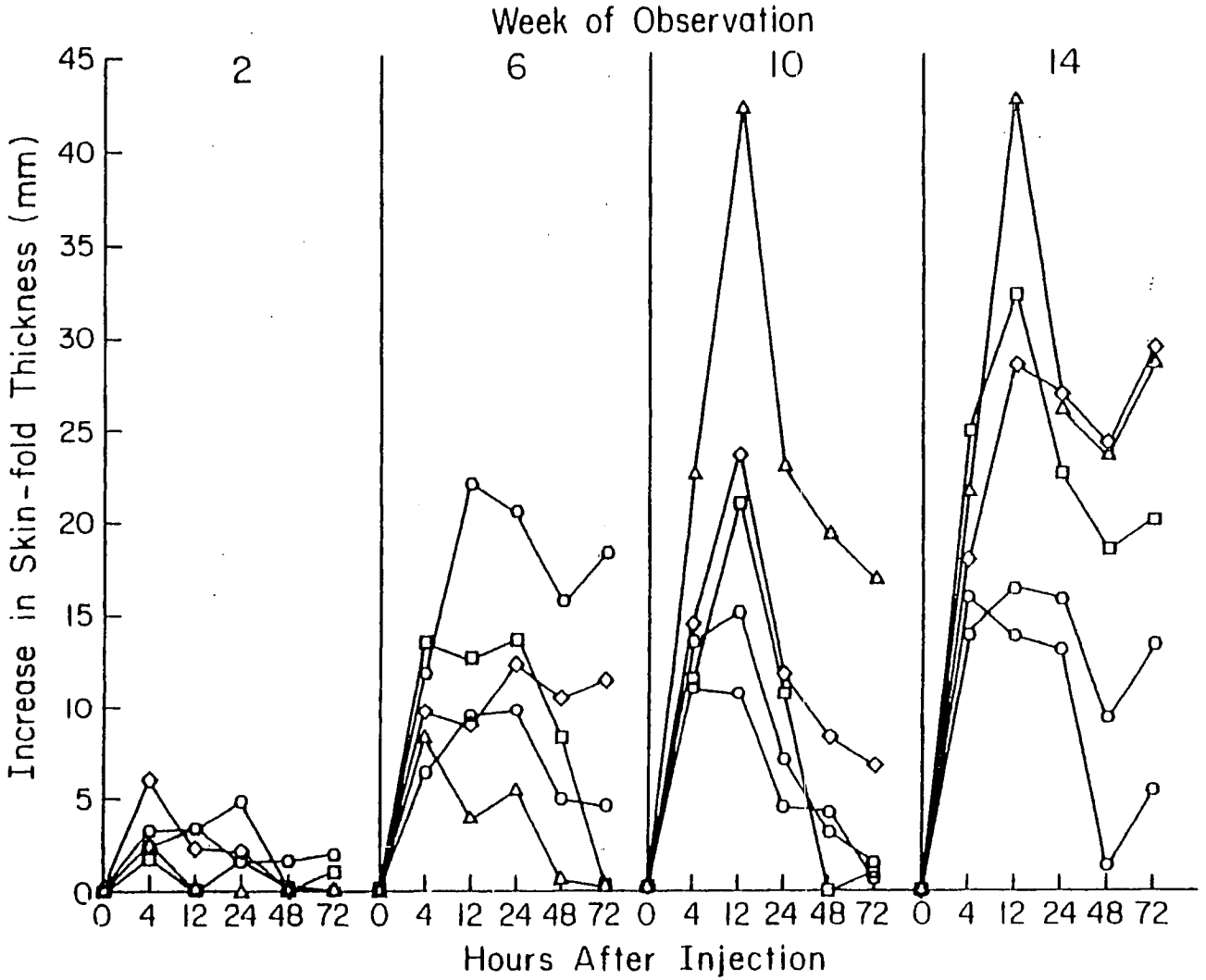
Week of Observation
2
6
10
14
Increase in Skin-fold Thickness (mm)
0
5
10
15
20
25
30
35
40
45
0 4 12 24 48 72 0 4 12 24 48 72 0 4 12 24 48 72 0 4 12 24 48 72
Hours After Injection

Figure 6. Increase in skinfold thickness measured in calves vaccinated with M bovis antigen in PBSS (○), FIA alone (△), and FIA with MDP (◇), Am-B (□) or Bp (⬡), and Challenge exposed to live organisms.

Table 1. Mean *in vitro* stimulation of pheripheral lymphocytes with phytohemagglutinin (PHA), conconavalin A (Con-A) and *M bovis*.

Lymphocytes from calves immunized with *M bovis* in:		Initial cultures stimulated with: PHA	Con-A	*M bovis*	Cultures at 14 weeks stimulated with: PHA	Con-A	*M bovis*
PBSS	$\bar{X}$	8.44*	17.46	0.62	22.72	25.06	0.67
(n = 3)	SD	7.16	10.69	0.64	24.96	22.75	0.71
Adjuvants:							
Freunds Incomplete	$\bar{X}$	10.12	16.21	0.30	33.72	42.99	0.62
(n = 3)	SD	11.20	12.14	1.10	45.47	57.25	0.50
Muramyl dipeptide	$\bar{X}$	12.29	40.16	0.42	29.56	49.22	0.72
(n = 3)	SD	13.93	29.46	0.16	18.93	24.94	0.72
Amphotericin-B	$\bar{X}$	14.49	47.52	0.33	29.26	44.05	0.53
(n = 3)	SD	14.54	26.27	0.15	26.37	43.09	0.23
Bordetella pertussis	$\bar{X}$	6.87	78.82	1.25	32.59	45.32	0.65
(n = 3)	SD	4.02	77.66	1.47	18.76	32.64	0.24
All animals	$\bar{X}$	10.44	40.03	0.58	29.57	41.33	0.64
(n = 15)	SD	10.52	43.58	0.76	26.86	36.58	0.49

*Stimulation index (see methods).

GENERAL CONCLUSION

A preventive approach must be employed in the control of bovine disease resulting from Mycoplasma bovis infection since no antimycoplasmal chemotherapeutic agent has proven satisfactory.[21,34] The two main preventive strategies, preventing contact between the virulent organism and the susceptible host, and inducing protective immunity through prophylactic vaccination, rely heavily on understanding the immunological responses to M. bovis.

New infections may result from direct or indirect contact with infected animals.[15,60] There are reports of experimental environmental contamination lasting a considerable time.[61,62] Preventing contact and transmission of infection requires identification and segregation of infected animals, coupled with strict santitation.[63] Control of spread between herds and the economic impact of the disease on a given farm are dependent on the speed and accuracy of detecting infected animals.

While the role of M. bovis in reproductive, joint, and respiratory disease is not well understood, and its economic impact is thus obscured, the role of M. bovis in mastitis has been well characterized and recently reviewed.[64,65] Bovine mycoplasmal mastitis is characterized by severe mastitis with prolonged loss of production. Early diagnosis in some cases has enabled control by slaugh-

ter with minimal loss. However, because of its contagious nature and easy transmissibility, mycoplasmal mastisis more often spreads rapidly within and between herds.[16] In 1977, 2.8% of California bulk tank milks were positive for pathogenic mycoplasma.[66] In 1981, 8.0% of bulk tank milks were positive, thus confirming concerns about rapid spread.[67]

The incidence and severity of M bovis mastitis is greater than that caused by other mycoplasmas. Since the problem is growing in magnitude, it is crucial to develop rapid, efficient, reliable and inexpensive diagnostic techniques suitable for use by diagnostic laboratories, veterinary practitioners and possibly dairy processors. The present methods of culture and identification of mycoplasma are time-consuming, require specialized equipment and technical skills, and may be unreliable.[35] Immunochemical techniques can be rapid, inexpensive, reliable and require minimal technical skills once they are perfected.[68]

Several features of the mycoplasmatales limit the utility of conventional reagents used in immunochemical diagnosis. Many mycoplasmas, including M bovis, are not potent immunogens and do not evoke production of avid and specific antiserum.[33,36,37] They incorporate components of the growth milieu in their membranes and, when used as antigens for production of specific antiserum, elicit reactivity to growth medium components as well as to innate

mycoplasma antigens, reducing specificity.[42,43,45] Numerous investigations substantiate the existence of shared proteins among a variety of mycoplasmas.[39,40,44,69-75] Two dimensional electrophoresis on other species have demonstrated that distinct antigenic complexes are not completely separated by SDS-PAGE,[69,70,76-78] and thus preparation of monospecific reagents from preparative gels will probably not be useful.

Analysis of M bovis antigens by GEDELISA, which combines the high resolving power of SDS-PAGE to separate molecules by molecular weight with the sensitivity of ELISA to detect specific antibodies[54,55] revealed at least 3 growth medium components which crossreacted with the six other species tested, two components which were non-specifically reactive, and 4 antigenic complexes which may contain unique M bovis antigens.[38] Species-specific antigens have been identified for a number of mycoplasmas.[69,71,72,78,79]

Hybridized cell lines have been adapted for productuion of species-specific, monoclonal antibodies to mycoplasma antigens and the sensitivity and specificity of these reagents compares well to conventional antisera.[80-82] These reagents would greatly facilitate worldwide distribution of identical antibodies for purposes of research and taxonomy. Their use would avoid discrepancies resulting from variation in mycoplasma strains used as antigens, from the confounding

effect of complex media used in their cultivation for use as antigen, and from differences in the immune responses of animals used to produce antisera. Highly sensitive and specific assay systems can be produced by combining the great sensitivity of immunochemistry for detection of antigens from infecting organisms with the great specificity of monoclonal antibodies.[83]

Immunochemical detection of M bovis with species-specific antibodies should be pursued as an attainable short-range goal. Rapid diagnosis of infected cows coupled with attentive management will greatly aid in controlling M bovis-related disease. This approach will aid in detecting infected animals within or entering a herd, and has potential for use in surveillance of bulk tank milk. Such a surveillance system utilized by the veterinarian, creamery, or dairyman could provide an early-warning for potential outbreaks of mycoplasmal mastitis.

The other main preventive strategy to control M bovis disease, that of prophylactic vaccination, also requires understanding the immunologic reponses to M bovis. Evidence supports the concept that vaccinated animals become immunologically resistant to experimental and natural M bovis disease.[19,25-30] Natural infection and vaccination, however, may sensitize animals to suffer hypersensitivity reactions on secondary exposure to antigen.[28,32,84] Thus vaccination with whole-cell, myco-

plasmal antigens may accentuate the disease.[85] If M bovis subunit antigens important in immunological resistance could be detected and isolated, their use as subunit vaccines might render vaccinated animals immunologically resistant yet less susceptible to harmful hypersensitive reactions.

The specificities of M bovis directed antibodies in sera from cows susceptible and cows resistant to experimental M bovis infection determined by GEDELISA were not statistically different.[56] The complexity of the M bovis antigenic collage discussed earlier may limit characterization of the response to important antigens by conventional means such as GEDELISA and two-dimensional chromatography.

Investigations characterizing M bovis antigens using different or refined methodologies should be pursued. Antigens which, when recognized, confer protective immunity should be described and isolated. In addition to their potential use as subunit vaccines, these antigens may be useful in differentiating animals which are serologically positive when tested with whole M bovis cell antigens but still susceptible to infection, from those serologically positive animals which are immunologically resistant. In addition to eliciting hypersensitivity reactions, M bovis has been associated with suppression of B-cell maturation in vivo,[84,86] and in vivo and in vitro lympho-

cyte blastogenisis.[86] Characterization of these events, as well as M bovis toxin(s)[87] and adhesion(s) would be of great interest. Antigenic dissection of M bovis will provide important information about the immunologic, microbiologic and pathologic events of the host-parasite interactions.

Despite the strong association between M bovis and disease,[13] the literature describes isolations of M bovis from healthy animals.[13-15] It was hoped that a study of the immunologic and microbiologic parameters of natural respiratory infection would serve as a baseline to compare vaccination and experimental challenge exposure, and lead to a better understanding of M bovis infection.

M bovis, like other respiratory mycoplasmas demonstrates a tissue distribution within the calf respiratory tract.[57-59] This may serve as presumptive evidence that a specific receptor-adhesion interaction occurs during respiratory infection which aids M bovis in avoiding the innate clearance mechanisms present in airways. The events and surface structures which participate in this interaction should be characterized as they have been for other respiratory mycoplasmas.[87-93] Changes in the respiratory distribution of M bovis with age or under varied immunologic conditions, and the importance of respiratory infection in providing a reservoir for M bovis and in developing

immunological resistance to disease should be considered further.

Measurements of immune responses in naturally infected calves indicate a high and prolonged serum IgM but low IgG and IgA response, subdued respiratory antibody response, low skin test reactivity, and low LST results compared with vaccinated calves. These results agree with other observations where comparisons can be made and support the hypothesis that mycoplasmas may interact with cells of the host's immunologic system.[28-33,84] Immunomodulation and absorption of antigens known to function in the hosts immune response which have been observed with other mycoplasmas[93-101] should be studied in M bovis.

More in vitro experimentation should be performed in order to develop and test hypotheses regarding the cellular interactions of M bovis infection as more bovine cell lines and antisera specific for BoLA or other important host antigens become available. Successful in vivo experimentation will depend directly on information on cellular interactions derived from such in vitro assays.

Humoral and cellular immune responses to M bovis vaccination can be enhanced using adjuvants.[31,32] Enhancing the local, respiratory immune responses to M bovis with adjuvants so that smaller amounts or subunit antigens can be used efficaciously will help to avoid undesirable hypersensitivity reactions.[85,102,103] None of the

adjuvants used in this study performed better in eliciting a mature humoral or cellular response to whole M bovis cells than FIA. No adjuvant enhanced the respiratory response above that found with antigen alone. Future studies will require understanding the excitation, migration, deposition, and memory of cells important in immunological resistance to M bovis disease. Understanding immunological resistance and developing a method to elicit a protective immune response will remain long-range goals in controlling M bovis disease.

Control of M bovis disease by vaccinal prophylaxis or identification/segregation of infected animals requires a clear understanding of M bovis antigens and host responses important in protective immunity. The work described here is a contribution to the repertoire of information, research tools and strategies which may be useful in achieving this goal.

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Antigen capture ELISA using a monoclonal antibody for the detection of *Mycoplasma bovis* in milk

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(Accepted 6 April 1993)

ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (mAb) was developed to detect *Mycoplasma (M.) bovis* in milk samples from cattle. With this procedure, 1×10^5 colony forming units per ml (cfu/ml) milk were routinely detectable. No cross-reactions to other bovine mycoplasma species were observed. Both the sensitivity of 80.6% and the specificity of 94.9% are sufficient for its use in diagnosis of clinical mastitis. The sensitivity could be increased by 10% after introduction of 48-hour pre-incubation of samples. This allowed recognition of cows shedding *M. bovis* amounts of 10^3 cfu/ml in their milk, which is typical for subclinical cases. Screening of milk samples by means of this antigen capture ELISA has advantages over culture methods in terms of speed and potential to monitor large herds, thereby permitting early culling of infected animals to reduce transmission of the pathogen to non-infected animals.

INTRODUCTION

Mycoplasma (M.) bovis is known to cause severe clinical mycoplasmal mastitis in cattle (Jasper, 1982). To control the spread of this disease an early detection of the pathogen is crucial since the removal and culling of infected cows is necessary to prevent fresh infections. Several ELISA procedures have been developed to detect serum antibodies against bovine mycoplasmas causing mastitis (Boothby et al., 1981; Thomas et al., 1987). Jurmanova et al. (1986) described the use of an ELISA for the detection of antibodies against *Mycoplasma* spp. and *Ureaplasma* spp. in milk. ELISA procedures using monoclonal antibodies (mAbs) have been developed to detect *M. bovis* antigen in semen and preputial washings (Nielsen et al., 1987), as well as in

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milk (Boothby et al., 1986). The assay described by Boothby et al. (1986) allowed the detection of 10^7 cfu of *M. bovis* per ml milk. Ball et al. (1990) described an antigen capture ELISA involving a mAb to detect *M. californicum* in milk samples. Using centrifugation for pre-enrichment of the agent, the sensitivity of this test could be improved from 10^7 to 10^6 cfu/ml milk.

In the present study, a fluorescence ELISA using a mAb produced in our own laboratory was developed as a screening test for the detection of *M. bovis* antigen in milk samples.

MATERIALS AND METHODS

Microorganisms

Table 1 contains the microorganisms used in this study. The *M. bovis* field strains were described previously (Sachse et al., 1992).

TABLE 1

Strains of *Mycoplasma (M.)* spp., *Acholeplasma (A.)* spp., *Streptococcus (S.)* spp., *Staphylococcus (St.) aureus* and *Escherichia (E.) coli* used in this study

Species	Denomination of strains
M. bovis	Donetta PG 45 (type strain), J 282, 221/89, 34/81, 422/88, 937/78, 365/88, 223/89, 108/79, 198/79, 222/89, 589/78 (field isolates)
M. bovigenitalium	PG 11
M. californicum	St-6
M. arginini	G 230
M. agalactiae	PG 2
M. alkalescens	PG 31/D 12
M. canadense	275 C
M. bovoculi	M 165/69
M. gatae	C 5
M. gallinarum	PG 16
M. bovine group 7	PG 50
M. mycoides subsp. *mycoides*	LC type, Y-goat
M. verecundum	St-107
M. gallisepticum	PG 31
A. laidlawii	PG 8
A. axanthum	S 743
A. modicum	PG 49
S. agalactiae	V 81/B 54, V 84/B 21 734
S. dysgalactiae	V 82/C 3151, V 86/C 8976
S. uberis	V 83/E 12 463
S. sp.	Lancefield group L
St. aureus var.* *bovis*	V 88, No. 67
E. coli	isolate from milk

*ecovar *bovis* and biotype *bovis* are also used for this organism.

Monoclonal Antibody

Preparation and properties of mAb Mb 4F6, which is directed against a 26 kDa protein of *M. bovis*, have been described elsewhere (Berthold et al., 1992). It belongs to isotypes IgG1 2b (heavy chain) and kappa (light chain).

ELISA

The antigen capture ELISA was carried out in ultramicrotitre format, which requires only 10-μl volumes of each reagent per well (Horn et al., 1982). The whole assay can, however, be conducted in the normal fashion with tenfold amounts of reagents. For coating, the wells were incubated with the ammonium sulfate-precipitated Ig fraction from ascites fluid (Peters and Baumgarten, 1990) of mAb Mb 4F6 in 0.05 M carbonate-bicarbonate buffer, pH 9.6, at 25°C for 4 h. Before applying milk samples to the microtitre plates they were preincubated using 1:2 dilutions of 0.01 M phosphate buffered saline (PBS), pH 7.2, containing 1% Tween 80 at 37°C for 15 min.

All further operations were carried out at 25°C in humid boxes. After coating with mAb Mb 4F6 the samples pretreated as above were added to the wells and incubated overnight. Each incubation step was followed by washing the plates once in 0.01 M PBS containing 0.05% Tween 20 and three times in distilled water. The plates were incubated for 4 h after addition of the biotinylated mAb Mb 4F6 (Hoffmann et al., 1982). Subsequently, AP-labelled streptavidin (Boehringer, Mannheim) was added and the plates were preincubated for 2 h before starting the substrate reaction involving 0.5 mM of the fluorogenic substrate 4-methylumbelliferyl phosphate (SERVA, Heidelberg) in 1 M diethanolamine buffer, pH 9.6. 0.05 mM 4-methylumbelliferone in 1 M diethanolamine buffer, pH 9.6, was used as standard value (fluorescence intensity 100) and 1 M diethanolamine buffer as blank. After the substrate reaction (approx. 25 min) the fluorescence intensity was measured using the photometer SUMAL PE 2 (ZEISS, Jena, Germany) at 365 nm.

Experimental samples

The test series included milk samples from cows experimentally infected with *M. bovis* (Pfützner et al., 1983), from natural outbreaks of mycoplasmal mastitis and cows having bacterial mastitis due to *Staphylococcus aureus*, *Streptococcus* spp. and *Escherichia (E.) coli*. The detection limit and specificity of the ELISA were evaluated using homologous (*M. bovis*) and heterologous bovine, as well as other animal and human mycoplasmas, acholeplasmas, streptococci, staphylococci and *E. coli* strains (cf. Table 1). All organisms tested were used as whole cells, and all milk samples investigated were also examined for mycoplasmas by conventional cultural methods. For verification of the ELISA, milk samples were cultivated in medium B and the number of mycoplasmas was determined by standardized methods (Gourlay and Howard, 1983). Mycoplasma isolates were identified by using the fluorescent

antibody agar block technique (Rosendal and Black, 1972). For pre-enrichment, 2 ml of each sample were transferred to a tube containing 2 ml of broth medium and incubated for 48 h. Simultaneously, another 2 ml of the milk sample were incubated without broth medium at 37°C. All samples were investigated by both ELISA and culture techniques.

RESULTS

With the present ELISA procedure, *M. bovis* was specifically detected from contaminated milk samples. The only cross-reaction observed was with *M. agalactiae*, whereas all other species named in Table 1 gave no signal (data not shown).

The calibration curve of *M. bovis* detection in artificially inoculated milk is presented in Fig. 1. From these data, a detection limit of 1×10^5 cfu/ml was determined. (A fluorescence intensity of twice the average background level was defined as a positive reaction.) No essential differences in reactivity between the various *M. bovis* strains were found. The calibration curve was practically identical for samples diluted in broth culture medium indicating the absence of non-specific reactions caused by milk proteins.

A total of 541 milk samples were investigated by ELISA and conventional culture techniques. 408 samples came from 9 experimentally infected cows,

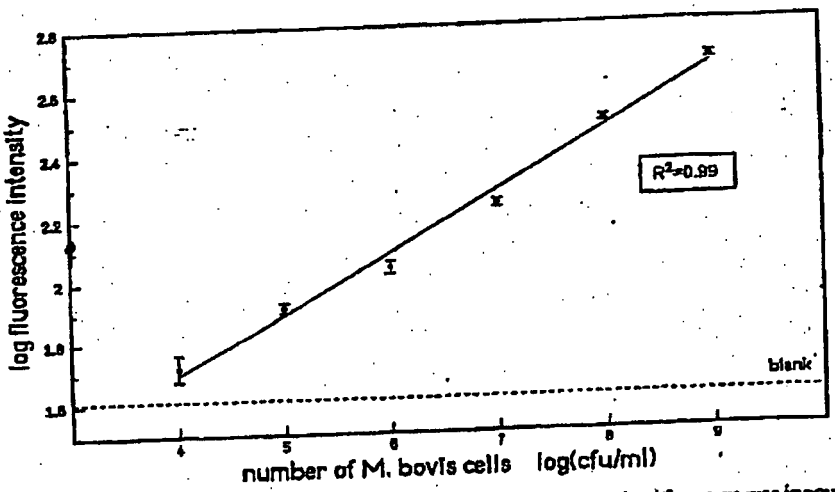

Fig. 1 ELISA calibration curve for *M. bovis* in milk. Raw milk from healthy cows was inoculated with different amounts of *M. bovis* cells, strain J 282, grown in broth culture medium (medium B, Freundt, 1983). 6 samples of each dilution were subjected to the ELISA. 0.05 mM 4-methylumbelliferone in 1 M diethanolamine buffer served as standard value (fluorescence intensity 100).

TABLE 2

Effect of pre-incubation on sensitivity and specificity of *M. bovis* detection by the antigen capture ELISA

	Number of milk samples	
	Without pre-incubation	With pre-incubation
true positives	166	39
false positives	17	7
true negatives	318	63
false negatives	40	4
sensitivity	80.6%	90.7%
specificity	94.9%	90.0%

113 from natural outbreaks of mycoplasmal mastitis and 20 from cows infected with non-mycoplasmal mastitis. From the last group, all samples were negative for *M. bovis*. The ELISA findings are summarized in Table 2, where the effect of sample pre-incubation on the performance of the ELISA is presented. Pre-enrichment of the pathogen by 48-hour incubation at 37°C as carried out with 113 milk samples resulted in an increase in sensitivity by 10.1%, while specificity was decreased by 4.9%. The addition of sterile broth medium to the milk samples prior to pre-incubation did not lead to further improvements of these parameters.

DISCUSSION

The results indicate that detection of *M. bovis* in milk using the present antigen capture ELISA is sufficiently specific. The cross-reaction of mAb Mb 4F6 with *M. agalactiae* does not affect the diagnosis of *M. bovis* mastitis in cows as *M. agalactiae* does not occur in cattle. Although Askaa and Erno (1976) assigned the respective type strains to different species there are indications that *M. bovis* and *M. agalactiae* share a considerable number of common antigens beyond the scope usually observed between distinct species (Berthold et al., 1992). Furthermore, the high degree of relatedness can also be derived from rRNA sequences (Mattsson et al. 1991). On the other hand, Boothby et al. (1986) and Nielsen et al. (1987) did not report cross-reactions of their mAbs with *M. agalactiae*.

The detection limit of 1×10^5 cfu/ml milk is sufficient for screening of cattle stocks with severe clinical *M. bovis* mastitis or high levels of *M. bovis* shedding. Pre-enrichment by 48-hour incubation enabled the detection of *M. bovis* at levels below 10^5 cfu/ml, which are typical for subclinical mastitis, latently and newly infected animals and cows persistently excreting the pathogen dur-

ing the chronical stage of disease. On these conditions, samples with original titres of 10^3 cfu/ml were recognized as positive by the ELISA. The increase in time required for *M. bovis* detection from milk samples due to pre-enrichment is still acceptable for routine diagnostics. Boothby et al. (1986) also included a pre-enrichment step in their competitive ELISA, which enabled them to raise the sensitivity from 65 to 86% at an *M. bovis* detection limit of 10^7 cfu/ml milk. Using an immunobinding assay involving the same mAb, Infante-Martinez et al. (1989) reported a further increase in *M. bovis* detection limits to $4.8–5 \times 10^3$ cfu/ml milk. However, because of its limitations with regard to automation immunobinding assays have not found widespread use in routine laboratories.

It was attempted to increase the sensitivity by coating plates with polyclonal antiserum and/or labelling the mAb with alkaline phosphatase. Although this resulted in a reduction of background by about 75% the detection limit was not improved since fluorescence intensities of positive samples were reduced by a similar extent.

The ultramicrotitre format ELISA used throughout this work requires only 10 μl of each reagent per well, which makes the procedure very economical. It is most practicable and sensitive as a fluorescence assay, but can be carried out in the normal fashion as well. The main advantage of the present ELISA procedure is the rapid detection of *M. bovis* directly in milk. Assay results are available at the latest on the third day after receipt. Pre-incubation of milk samples to attain high sensitivity remains the limiting step. For comparison, conventional detection by culture methods would take about 5 days, sometimes up to 10 days.

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Mycoplasma
PART 1

Inventor: Leonard *et al.*
Title: Vaccines for Mycoplasma Bovis and Method of Use
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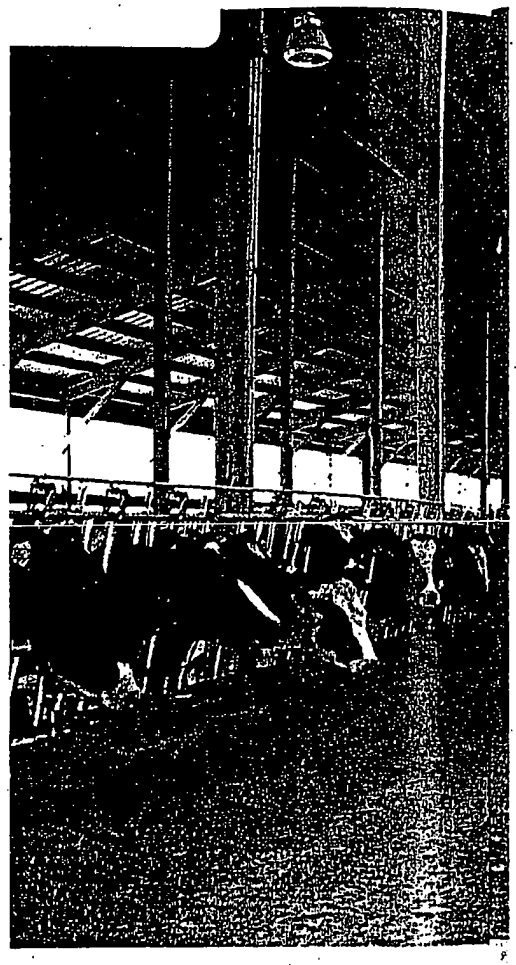
Mycoplasma mastitis: It's everyone's problem



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***Mycoplasma* mastitis is a growing challenge on dairies across the U.S.**

By Maureen Hanson

Editor's note: First of two parts on Mycoplasma *mastitis.*

It once was considered strictly a "California" disease that only affected very large herds. Now it's on the rise throughout the country, and may soon be knocking on your own door, if it hasn't already. What is this "new" disease challenge that actually has been around for decades? *Mycoplasma* mastitis.

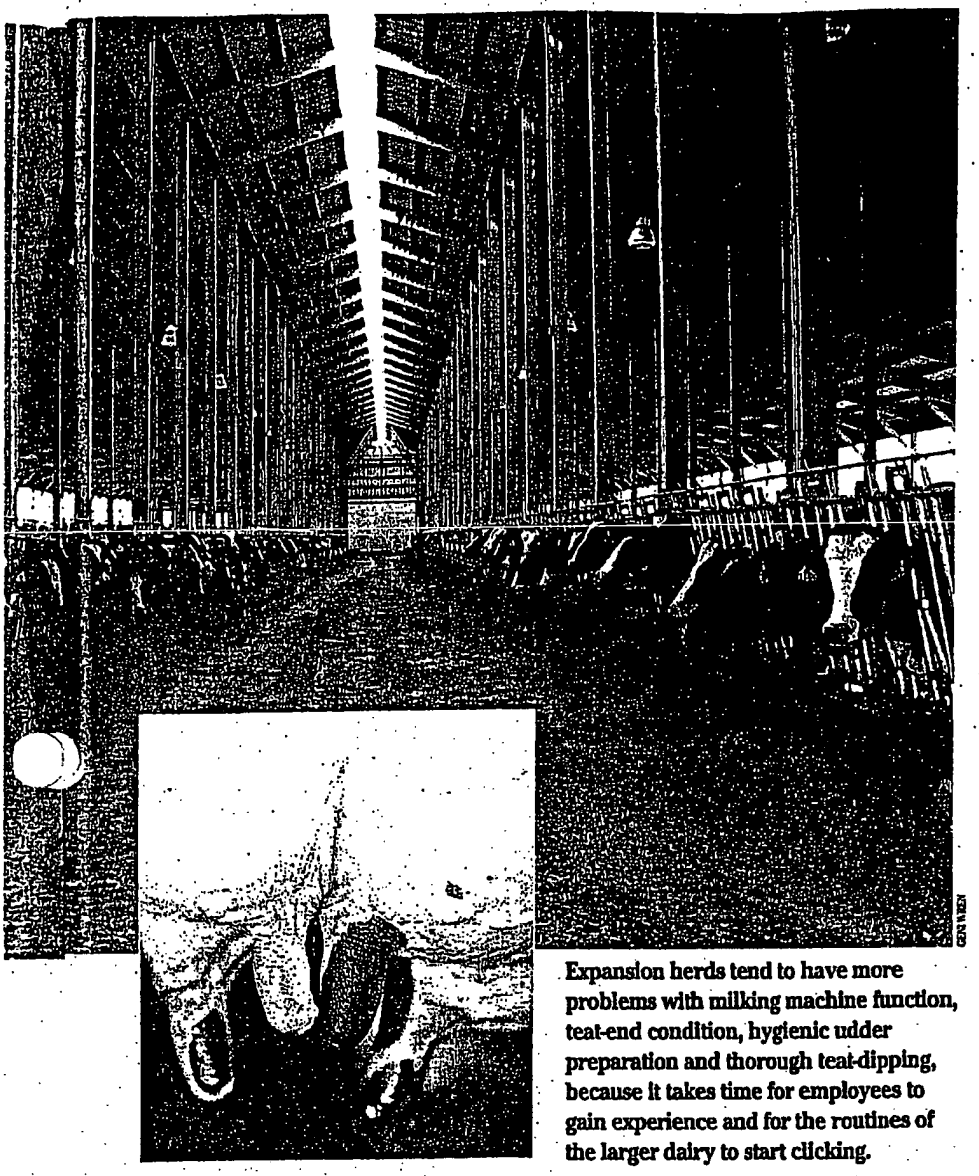
Veterinarians who have dealt with *Mycoplasma* first-hand can attest to the dread that accompanies its clinical emergence. One of them who has more than three decades of experience with it is Paul Blackmer, DVM, owner of Veterinarian's Outlet, Chino, Calif.

"*Mycoplasma* mastitis is a doubly insulting disease," says Blackmer. "Not only can it be remarkably contagious when it is present, but it absolutely does not respond to antibiotic therapy. In fact, treatment can actually cause epidemics, because it frequently is spread by unsound intramammary therapy practices. It's a problem that dairy producers must manage their way out of, because they can't treat their way out."

Client education required

Blackmer believes that helping clients understand the serious nature of *Mycoplasma* mastitis is a fundamental obligation of all practicing dairy veterinarians. "Preventing *Mycoplasma* outbreaks requires consistent adherence to specific management steps, on the part of both the owner and the individuals who manage the cows hands-on," he states. "Everyone must understand the negative consequences that a breakdown in the system could cause."

Those consequences can include non-responsive, clinical mastitis; a dramatic drop in individual-cow milk production; loss of single quarters that can advance to complete cessation of lactation; and — worst of all — rampant spread of clinical mastitis from cow to cow.



Expansion herds tend to have more problems with milking machine function, teat-end condition, hygienic udder preparation and thorough teat-dipping, because it takes time for employees to gain experience and for the routines of the larger dairy to start clicking.

What's more, the mammary system isn't the only area that can be affected by *Mycoplasma*, according to Mark Wustenberg, DVM, Technical Services Specialist, Monsanto Dairy, Bay City, Ore. A former practicing veterinarian who focused heavily on milk quality, Wustenberg says respiratory disease and/or arthritis also can plague *Mycoplasma*-infected animals – usually heifers.

The outlook becomes even grimmer when the effect of the disease on calves is weighed. "Calves that are fed *Mycoplasma*-infected milk can contract inner ear and respiratory infections, swollen joints and generalized septicemia that may become active as clinical mastitis upon freshening in first-calf heifers," says Wustenberg. (See Case Study 3 on page 46 of this issue).

Just how bad can it get? Within the past year, Blackmer has seen more than one dairy lose over 100 cows to clinical *Mycoplasma* mastitis in less than a month's time. "Make no mistake," says Blackmer, "this disease is a very real

Continued on page 6

Mycoplasma outbreak examples

The circumstances that lead to a *Mycoplasma* mastitis outbreak vary greatly. Following are recent examples of cases observed by Mark Wustenberg, DVM:

- A herd that switched from teat dipping to teat spraying, resulting in poor coverage of the teats with disinfectant.
- An expansion herd that was having difficulty getting new animals to use the parlor. The milkers shifted their efforts from good udder hygiene to moving cows — which already were affected by commingling and stress — through the parlor.
- A herd that started using a new teat dip that was difficult to see on the teats, at about the same time that a new batch of heifers was brought in. Teat dipping was sometimes skipped because of the problem with dip color.
- A herd that was diluting medication with hypertonic saline and then infusing it intramammarily.

threat to the economic stability of a dairy."

Changing landscape fuels spread

When *Mycoplasma* mastitis was first identified and diagnosed in the 1960s, clinical cases were confined mostly to the then large-herd dairy states like California and Florida. Blackmer attributes this to the owner-milks-cows versus labor-milks-cows phenomenon. "When herds in most of the country were small enough that the owner did most of the milking, *Mycoplasma* was kept well in check, because owners tended to identify and deal with problem cows quickly," he explains. "In larger herds, where employees do the milking and parlor through-put is a major priority, clinical cases can go undetected longer, and breakdowns in standard milking procedures are more likely to happen."

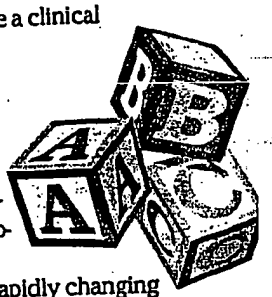
Clinical experience has shown that *Mycoplasma* outbreaks occur most frequently during times of stress, such as calving, extreme weather shifts and periods of commingling with new animals.

Allan Britten, DVM, MS, owner of Udder Health Systems, Inc., Bellingham, Wash., says recent consolidation and expansion of herds across the country have created conditions that are ripe for *Mycoplasma*a outbreaks. He and the other experts offer these explanations why:

■ **Animal stress.** *Mycoplasma* bacteria — particularly *M. bovis* — are endemic to the bovine respiratory and reproductive tract of many outwardly healthy cows and calves. Clinical experience has shown that *Mycoplasma* outbreaks occur most frequently during times of stress, such as calving, extreme weather shifts and periods of commingling with new animals. It is believed that the opportunistic organism takes hold when cows' immune systems are compromised. Plus, overcrowding increases the likelihood that *Mycoplasma* bacteria could travel from the respiratory or reproductive tract of one animal to the mammary system of another.

■ **Animal movement.** *Mycoplasma* often is introduced when replacement animals — particularly heifers — are commingled into a herd. Heifers are frequent carriers because they often are raised away from the dairy in "high-traffic" facilities and commingled with other animals, then shipped to the dairy for freshening. If heifers were fed *Mycoplasma*-infected milk, there also is the chance that they will harbor systemic *Mycoplasma* infections that break at calving. "Today there's almost no such thing as a 'closed herd,'" says Britten, adding that breeding bulls are another potential source of herd contamination.

■ **Milking practices.** The principles of good milking hygiene are fundamental to preventing the spread of *Mycoplasma* from cow-to-cow once a clinical



Mycoplasma fundamentals

The story of the organisms that cause *Mycoplasma* mastitis continues to unfold, via research and clinical impressions. Following is a summary of what is currently known about the bugs and the infections they cause.

■ ***Mycoplasma* mastitis** was first reported in the United States in New York in 1962. Much of the early research on the disease was performed by D.E. Jasper at Cornell University.

■ **There are at least** nine strains of *Mycoplasma* that have been isolated from milk. Not all are pathogenic for mastitis:

- *Mycoplasma alkalescens*
- *Mycoplasma agrinini*
- *Mycoplasma bovigenitalium*
- *Mycoplasma bovirhinis*
- *Mycoplasma bovis* (responsible for 50% or more of *Mycoplasma* mastitis infections)
- *Mycoplasma californicum*
- *Mycoplasma canadense*
- *Mycoplasma capricolum*
- Untypeable *Mycoplasma*

■ ***Mycoplasma* organisms** have no protective cell walls and minimal genetic material. They are resistant to antibiotics in clinically infected cows. It appears that the organisms are capable of rapidly changing their surface proteins. An animal's immune system targets disease-causing bacteria for destruction by locking onto the shape and size of the bacteria's surface structures. In the case of *Mycoplasma*, these structures keep changing, the immune system is confused and the organisms escape.

■ **Infected animals are** the single most important reservoir of *Mycoplasma* species that cause mastitis.

■ **Herds with and without** clinical *Mycoplasma* mastitis may include both young and mature asymptomatic carriers. The organism may be shed in nasal discharges of calves and vaginal discharges of heifers at calving.

■ ***Mycoplasma* bacteria** are capable of surviving (but not growing) in milk (60 days), air, manure (236 days), urine, feed, straw, water, etc.

■ **It has been shown** that the vaginal discharges and urine of cows after calving are greatly contaminated with several *Mycoplasma* species. Milking teats contaminated with vaginal discharges and urine could likely lead to clinical mastitis.

mastitis infection is established. Expansion herds tend to have more problems with milking machine function, teat-end condition, hygienic udder preparation and thorough teat dipping, simply because it takes time for employees to gain experience and for the routines of the larger dairy to start clicking.

Hospital management. Start-up and expansion dairies often relegate the hospital to one of the last structures to be completed. This leaves substandard or no facilities for segregating clinical mastitis cows, allowing *Mycoplasma*-infected animals to spread the infection to their herdmates.

Continued on page 8

Mark Wustenberg, DVM, says infusing anything into the udder from a bottle is almost a guaranteed method of spreading *Mycoplasma*.

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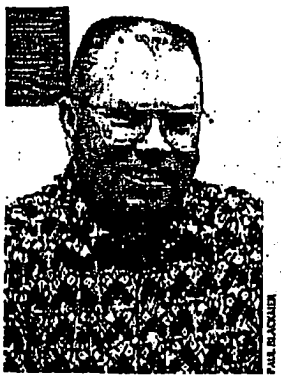


Paul Blackmer, DVM, says *Mycoplasma* is a threat to the economic stability of the dairy.

Once established in the milking herd, infections are most often spread during milking or mastitis cannula infusion.

Intermittent shedding by asymptomatic carriers may interfere with the accuracy of screening milk from incoming animals.

Cows of all ages and at any stage of lactation are susceptible to *Mycoplasma* mastitis infections. However, cows in early lactation seem to suffer more frequently and severely because the infection may be enhanced by existing inflammatory processes in fresh-cow udders. Clinical signs include:

- Sudden onset of edematous swelling of the udder.
- Severe clinical mastitis that resists treatment, but produces little other effect on the cow. Affected animals continue to eat and drink normally, although the udder tissue may be painful.
- Involvement of more than one quarter and up to all four.
- A marked drop in milk production and increase in individual SCC.
- Abnormal udder secretions that may vary from watery milk with a few clots to a thick, purulent material in advanced cases. Acutely infected cows may show a tannish secretion with sandy or flaky sediments that resemble cooked cereal in a whey-like fluid. Udder secretions may become purulent and last for several weeks.
- Swollen, meaty udders that do not involute properly during milking.

In most cases, infections become established via invasion of the mammary system through the teat orifice. The exception to this may be systemically infected heifers that were infected as calves.

From 10 to 50% of cows may return to a high level of milk production after surviving clinical *Mycoplasmaa* mastitis during a previous lactation but may continue to be intermittent shedders of *Mycoplasma* organisms in their milk for the rest of their productive lives.

Lameness due to arthritis caused by the presence of *Mycoplasma* in the hocks and fetlocks of mastitic and nonmastitic cows is frequently seen in infected herds. Arthritic involvement indicates probable long-term presence of the bacteria in the animal.

The organism is very sensitive to the pH changes in milk. Best recovery rates are achieved when fresh milk samples are delivered to the lab and plated soon after collection. However, samples also can be refrigerated for up to three days, and freezing milk samples does not interfere appreciably with the ability to culture *Mycoplasma*.

It appears that any germicide that effectively controls *Staph. aureus* will control *Mycoplasma*. Because iodine disinfectants and sanitizers appear to be the most effective against both *Staph. aureus* and *Mycoplasma* organisms, it is recommended that iodine be the sanitizer of choice in a *Mycoplasma* outbreak.

Sources:

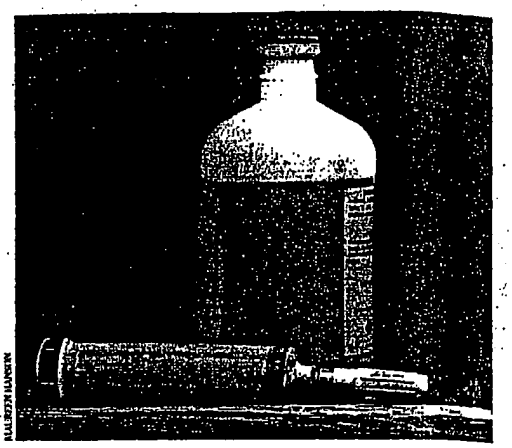
- Bushnell, R.B., *Mycoplasma* Mastitis, Symposium on Bovine Mastitis, *Veterinary Clinics of North America: Large Animal Practice*, Vol. 6, No. 2, July 1984.
- Gonzalez, R.N., Mycoplasma Mastitis in Dairy Cattle: If Ignored, It Can Be a Costly Drain on the Milk Producer, *Proceedings of the 1996 National Mastitis Council Regional Meeting*, pp. 37-44.
- Kirk, J.N. and Lauerman, L.H., *Mycoplasma* Mastitis in Dairy Cows, *The Compendium of Clinical Education in Veterinary Medicine*, Vol. 16, No. 4, April 1994.
- Kirk, J.N., *Mycoplasma* Mastitis: Overview, University of California-Davis, Veterinary Medicine Extension Service, Veterinary Medicine Teaching and Research Center publication, Tulare, Calif.
- Rosenbusch, R.F., U.S.-Israeli Scientists Target Cattle and Sheep Diseases, Iowa State University Office of Biotechnology publication, Ames, Iowa.

These factors explain why more *Mycoplasma* cases are creeping into other parts of the country. In Wisconsin alone, the incidence of clinical *Mycoplasma* mastitis outbreaks has climbed from just two herds in 1992 to 83 herds in 2000, with a total of approximately 319 herd outbreaks during that time span (see Figure 1). Similarly, herds in the Pacific Northwest (mostly in Washington) have been experiencing a noticeble increase in the presence of *Mycoplasma* from just 18 positive herds in 1993 to 126 in 2000 (see Figure 2). "It's not that we just started screeening for *Mycoplasma* and began to find it," says Britten. "We've been looking for it consistently, and we're truly seeing more of it."

"Usually, when a *Mycoplasma* outbreak occurs, it's because something has broken in the regular routine," says Wustenberg. "It takes a while for start-up and expansion dairies to establish routines, which is how *Mycoplasma* flares up."

Eliminating one unsound management practice, however, can protect dairies from taking unnecessary risks with the disease. "Infusing anything into the udder from a bottle is almost a guaranteed method of spreading *Mycoplasma*," says Wustenberg. "Once a bottle is contaminated, it becomes the perfect reservoir for the organism to proliferate. Injecting home brews, saline or anything else from a bottle into the udder is a recipe for disaster."

Blackmer adds that a misguided treatment practice is infusing dexamethasone along with intramammary antibiotic therapy to reduce udder-tissue swelling. "One complaint we hear frequently about undiagnosed, clinical *Mycoplasma* cases is that the udder seems full but the milk won't come out," he explains. "That's because it is the blood vessels and supporting structures in the parenchyma — not the milk-storing alveoli — that become inflamed in *Mycoplasma* infections. The short-term result of this treatment is that the swelling goes down, but accompanying antibiotic therapy is completely ineffective, and the course of the disease remains unchanged. In the meantime, the bottle of dexamethasone often is contaminated with *Mycoplasma*. It is an ill-advised and completely irresponsible practice." (See Case Study 2 on page 46.) ■

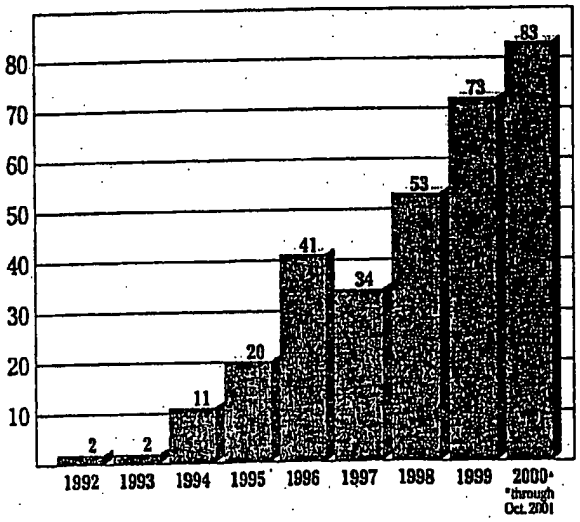


Injecting home brews, saline or anyting else from a bottle into the udder is a recipe for disaster

Next issue: Mycoplasma diagnosis, prevention and control.

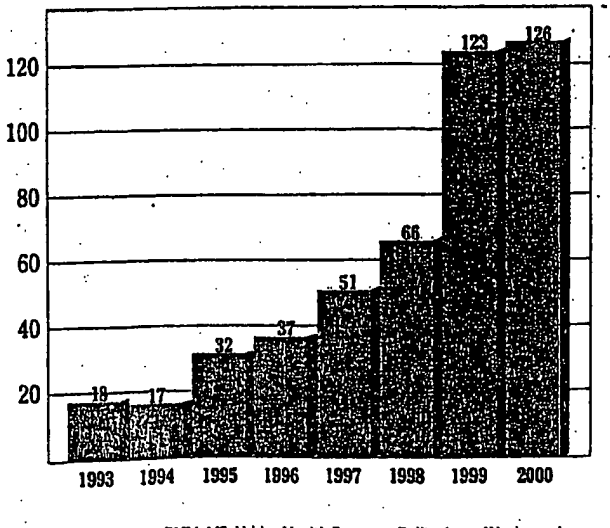
Mycoplasma mastitis is a propagating epidemic

Figure 1. Number of new Wisconsin herds with *Mycoplasma* spp. recovered from milk by year.



Source: Chet B. Thomas, DVM, epidemiologist at the University of Wisconsin School of Veterinary Medicine, Madison

Figure 2. *Mycoplasma* detected in dairy herds by bulk tank culture in Pacific Northwest dairies.



Source: Allan Britten, DVM, MS, Udder Health Systems, Bellingham, Wash./and Larry Fox, DVM, PhD, Washington State University, Pullman, Wash.

Inventor: Leonard *et al.*
Title: Vaccines for Mycoplasma Bovis and Method of Use
Atty Name: Gwendolyn D. Spratt
Atty Docket No.: 02108.0001U2 Serial No. 09/708,352
3 of 5 (6-Pages)

Mycoplasma
PART 2

Mycoplasma mastitis: Prevention and control

Mycoplasma mastitis could strike in any dairy operation in the country. Leading industry experts offer advice on how to be prepared.

By Maureen Hanson

Editor's note: Second in a two-part series on Mycoplasma *mastitis.*

Accurate diagnostics are a critical element in detecting and diagnosing *Mycoplasma* mastitis. Before you can effectively help clients prevent and control the disease, you need to be confident that *Mycoplasma* is, indeed, the organism with which you are dealing.

Mark Wustenberg, DVM, Monsanto Dairy, Bay City, Ore., and Paul Blackmer, DVM, Veterinarian's Outlet, Chino, Calif., emphasize the importance of sampling clinical cows and culturing them on both blood agar and *Mycoplasma* agar. As *Mycoplasma* becomes a larger concern across the country, Wustenberg recommends that practitioners check out the diagnostic labs they are using, to make sure they can accurately perform cultures for *Mycoplasma* and get consistent results.

"Visit the lab personally, and make sure they do a high volume of milk samples on a regular basis," says Wustenberg. "They should be able to show you their techniques, and particularly demonstrate their quality control measures. Finally, you should ask to see some examples of *Mycoplasma*-positive cultures and verify that the lab routinely cultures for *Mycoplasma* in milk samples, not just swine and poultry."

Blackmer notes that there is a misconception in some regions that *Mycoplasma* is hard to grow in the lab. It isn't. He believes the misinformation stems from labs using an inappropriate media in the early days of *Mycoplasma* screening.

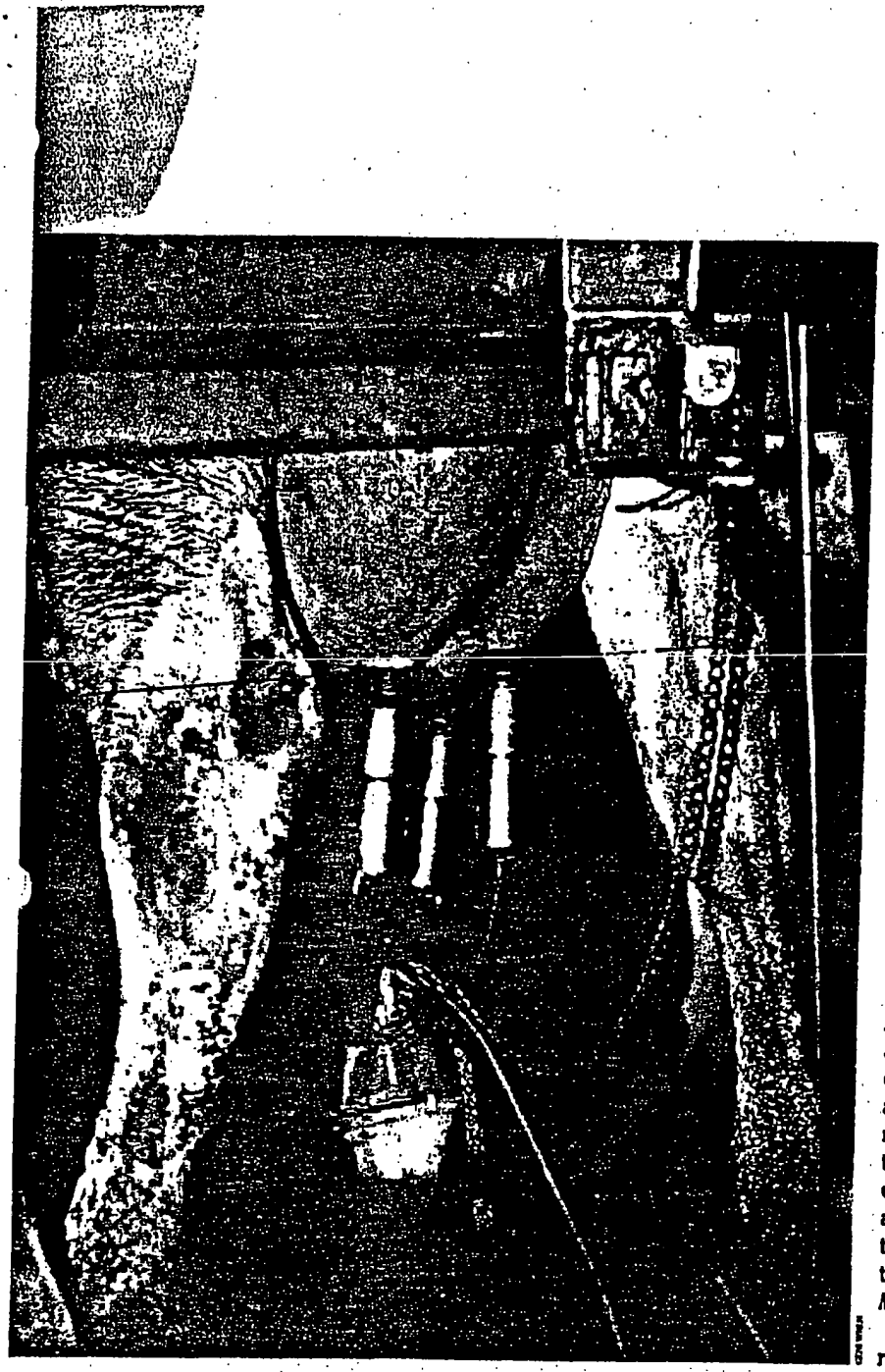
Many labs read *Mycoplasma* results at three and seven days of growth, so this timeframe must be factored into the decision-making process in outbreak management. If *Mycoplasma*

What's a practitioner to do?

Following are basic, recommended measures by Paul Blackmer, DVM, which every dairy veterinarian can follow to help all dairy clients protect their herds from *Mycoplasma* mastitis:

- Culture every clinical quarter of every clinical cow on *both* blood agar and *Mycoplasma* media.
- Routinely screen bulk tank samples for *Mycoplasma*.
- Sample and culture every fresh heifer and purchased animal.
- Educate milkers on hygienic milking and mastitis-tube infusion practices.
- Prohibit all use of bottle-sourced intramammary infusions.

For a primer on aseptic milk sampling procedures, visit Blackmer's website at **www.vetoutletonline.com.**



Inadequate teat dipping, milking dirty cows and poor claw sanitizing can help spread *Mycoplasma*.

growth is detected in a new herd with no *Mycoplasma* mastitis history, Wustenberg also recommends the additional step of validating the sample to confirm that it is *Mycoplasma* and speciating the organism to determine which strain is involved.

Today, some labs offer enrichment — preincubating milk samples in a broth to enhance *Mycoplasma* growth, then plating samples from there — to provide extra insurance that *Mycoplasma* is detected. Although this procedure was developed to better detect and control a dangerous organism, Blackmer strongly believes that it actually clouds the diagnostic picture.

"The more we learn about *Mycoplasma*, the more we know that the organism is everywhere in the dairy cow's environment," he explains. "The chances of pulling a few *Mycoplasma* bugs from the air into a milking system are great, even with impeccable sampling techniques. So enrichment will allow them to grow, creating false-positive results and diagnostic overreacting."

Unlike Blackmer, Wustenberg sees value in enriching bulk-tank samples. "We did extensive work on paired cultures to compare the results of enriched and non-enriched bulk-tank milk and found multiple instances where we detected low levels of positive cows after enrichment," he notes. "The false-positives at the bulk-tank level we found were few, if any. The trick is to recognize that there is the potential for false-positives, and develop a strategy to confirm before panicking."

But Udder Health Systems, Inc., Bellingham, Wash., owned by Allan Britten, DVM, MS, processes thousands of *Mycoplasma* diagnostic samples from across the country each year, with no enrichment ever used. "I have not seen any proof that enrichment yields a significant number of additional, true positive samples that would provide better control of the disease," says Britten. "It adds time and expense to the diagnostic process and is not the most important variable in the management of *Mycoplasma* mastitis."

Blackmer's advice instead: "Do direct plating of all milk samples onto *Mycoplasma* agar. If you have an intramammary infection, it will show up loud and clear."

Prevention strategies

The highly contagious nature of *Mycoplasma* makes biosecurity an essential element in keeping the disease in check.

Four groups of animals are at an exceptionally high risk of either contracting *Mycoplasma* mastitis or spreading it within the herd:

1. Incoming first-calf heifers

Mycoplasma
PART 2

Continued from page 13

2. Other newly acquired animals
3. Fresh cows
4. Sick cows entering or leaving the hospital for any reason

There are a number of practical measures that the experts recommend to prevent *Mycoplasma* mastitis from entering and spreading in a herd:

■ **Dedicating separate, sanitary facilities for maternity and sick cows** — versus mixing them together or leaving them with the main herd — will help cut down on the amount of cross-contamination when a clinical case occurs. Ideally, the hospital area should have its own milking facility for fresh and sick cows. If this is not practical, fresh cows and the hospital string should be milked separately from the rest of the herd, with thorough sanitation of the milking clusters after they are milked.

■ **Culturing every cow as she leaves the hospital** will help identify new infections that may have occurred in the hospital, and keep a potential outbreak in check.

■ **Quarantining incoming groups of heifers or individual animals** and culturing composite, four-quarter samples before releasing them, will help prevent newcomers from seeding the rest of the herd with *Mycoplasma*.

■ **Regularly sampling the bulk tank and culturing for *Mycoplasma*.** Britten suggests that bulk-tank samples be taken and cultured as frequently a once a week in herds larger than 1,000 cows and those with a history of *Mycoplasma* mastitis. Sampling monthly or every other week may be appropriate for smaller herds with no previous *Mycoplasma* episodes.

■ **Scrupulous udder hygiene** — including thorough pre- and post-dipping of teats, using individual cloth towels and maintaining excellent teat-end condition — also will prevent the spread of *Mycoplasma*.

■ **Good teat cannula management** also cannot be overemphasized. "Poor treatment hygiene can turn an isolated clinical *Mycoplasma* case into a massive outbreak virtually overnight," says Blackmer. "Even having more than one treatment cannula exposed in the hospital or parlor is risky, given the very high likelihood that it could be seeded with *Mycoplasma* from the air, flies, fingers, swishing tails, splattering urine or uterine discharge before it enters the teat orifice."

■ **Culturing every clinical quarter of every cow with clinical mastitis** will help producers pinpoint *Mycoplasma*

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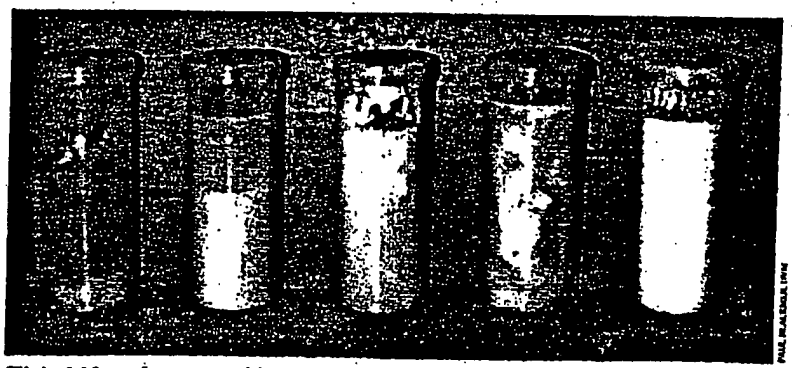
Poor treatment hygiene can turn an isolated clinical *Mycoplasma* case into a massive outbreak virtually overnight.



Clinical *Mycoplasma* mastitis often produces severely altered mammary secretions that appear tannish in color with hard, gritty clots.

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cases and deal with them knowledgably. Wustenberg also suggests monitoring individual-cow SCCs and culturing cows that convert from an SCC below 200,000 to above 200,000 in a one-month period.

■ **Culturing composite milk samples from all fresh heifers** (and also fresh cows if the herd has a history of *Mycoplasma* mastitis) also is advised.

The subject of backflush units also stirs some debate among the veterinarians. "Backflush units can easily create a false sense of security that causes a dairy to cut corners on the other fundamental practices that are important for effective *Mycoplasma* control," states Blackmer. "They also tend to use an excessive amount of water and chemicals, and often the valves don't stay clean. They can cause deteriorating milk quality, and their external surfaces remain fomites."

But Wustenberg and Britten aren't so quick to cancel out the technology. Wustenberg likens backflush to a rider on an insurance policy. "You can buy different levels of insurance, and some dairymen are more risk-averse than others," he says. "Backflush does not replace good, basic management practices, but, when used and maintained appropriately, it can be part of the solution."

Britten adds that, like virtually any technology, backflush can be useful but only when it's used appropriately. "It certainly doesn't make any sense to put a dirty machine on a healthy cow," he says, "but if backflush is going to be used for protection, it has to be functioning effectively and safely. It is an important role for the veterinarian to provide counsel to clients and communicate with equipment dealers and technicians. The practitioner should be regularly evaluating the system for alignment, leakage and germicide coverage and pointing things out that need improvement."

Outbreak offense

When a routine bulk-tank culture shows positive for *Mycoplasma*, the best-case scenario is that it amounts to no more than a single, culprit cow being identi-

One-day *Mycoplasma* diagnosis?

One of the challenges of managing *Mycoplasma* mastitis outbreaks to date has been waiting out the three-to-ten-day period that it took to achieve precise culture results. Those days may soon be over, with the advent of polymerase chain reaction (PCR) technology.

PCR is the quick method for generating unlimited copies of any fragment of DNA. Sometimes referred to as "molecular photocopying," it can characterize, analyze and synthesize any specific piece of DNA or RNA. This exciting new technology is less than 15 years old.

Udder Health Systems, Inc., Bellingham, Wash., is one of the first mastitis diagnostic labs in the country equipped to perform PCR. Owner Allan Britten, DVM, MS, says PCR is a breakthrough method that could dramatically speed up the process of diagnosing *Mycoplasma* mastitis. "Using current methods, we don't declare a sample *Mycoplasma*-negative until a full 10 days after culturing," says Britten. "The hazard with that timeframe is that potentially infected animals are not dealt with until the results are back. Because *Mycoplasma* sheds in such high numbers from infected animals, every day is critical in making a decision about those cows."

Britten and his staff are currently conducting an in-house research project to evaluate the sensitivity and specificity of PCR compared to standard culturing for *Mycoplasma* organisms. Although the outcome of such a comparison could vary from one diagnostic lab to the next — depending on the methodology used — a favorable outcome for PCR could mean much more efficient *Mycoplasma* diagnostics in the future.

"PCR results for *Mycoplasma* can be obtained in four to five hours, so a lab could deliver diagnostic results virtually overnight," says Britten. Another appealing feature about the technology is that, when done with proper controls, PCR eliminates confusion with a confounding *Mycoplasma* look-alike. "When screening milk for *M. bovis* DNA with PCR, you don't have to worry about getting false-positive *Acholeplasma laidlawii* results," he notes.

The test is flexible, in that it can be manipulated to screen for *Mycoplasma* species in general or for individual, specific strains. Individual tests can be performed for speciation of each strain. PCR speciation can be done on isolated colonies off *Mycoplasma* agar or directly from milk before isolation.

In fact, Britten is so confident in the accuracy of PCR for *Mycoplasma* speciation that it is the one service for which his lab currently offers PCR on a commercial basis.

Britten says his staff has embraced the method fairly quickly, noting that new microbiology graduates are now trained on PCR methods in school. "The more 'seasoned' technologists among us have had to go back to the laboratory classroom for PCR training, but it is not a difficult technique to learn," he says.

Although the equipment and related accessories needed to perform PCR are becoming less expensive, Britten says cost is still a concern. Labs interested in adding PCR services should allocate a start-up budget of $30,000 to $40,000. He says the per-test cost still hovers over $15, but that he hopes the technology will continue to become more economical.

Udder Health Systems routinely performs diagnostic work for veterinary practices across the country.

For more information on PCR, contact Udder Health Systems, Inc., 6401 Old Guide Road, Bellingham, WA 98226, 360-398-1360, udder@az.com.

Continued from page 16

fied and culled. A positive tank alone does not automatically spell disaster, Wustenberg points out, because in this scenario the problem can be identified and eradicated very swiftly.

Detecting the perpetrator(s) may require various levels of investigation:

- In some cases, a new heifer or clinical cow immediately sticks out and is found, confirmed and culled. Such animals will be even more obvious on dairies where routine cultures are performed on every cow undergoing intramammary therapy.
- The next level of investigation is string sampling: culturing composite samples from milking strings and then performing individual-cow cultures only on the animals in the positive string(s).
- In cases with heavy bulk-tank *Mycoplasma* loads (heavy growth) and/or a herd history of *Mycoplasma* mastitis, whole-herd culturing to detect the positive cow(s) may be warranted.

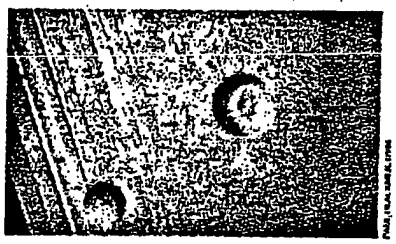
The other way that *Mycoplasma* typically surfaces in a herd is a high incidence of nonresponsive mastitis cows in the hospital. Wustenberg says that in those cases, the bulk tank may never even go positive, because clinical mastitis milk is withheld from the tank, and the *Mycoplasma*-positive cows are either culled or identified via culturing of clinical quarters. Or, the incidence of infected cows is low enough that it dilutes out in the bulk tank.

What to do with *Mycoplasma*-positive cows is another issue of some debate in the industry. If the problem stems from one or two cows, culling is deemed the most appropriate measure. Likewise, no one believes that chronic clinical cows should be kept around.

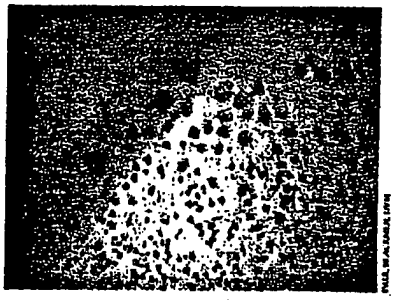
But in the case of larger-scale outbreaks, "wholesale culling may be an extravagance that some dairies cannot afford," says Blackmer. This is particularly true in expansion situations, when the dairy is under pressure to maintain cow numbers for collateral.

One diagnostic concern is the cow that cultures *Mycoplasma*-positive but exhibits no clinical signs. Given the high number of colony forming units (CFUs) in a true clinical case, sample contamination can be a common problem. Blackmer describes a "trailer effect" that poor sampling technique can produce: "In three samples taken in a row, the first cow may be clinical and show obvious, confluent growth. The next one may show moderate growth, and the third one a trace of *Mycoplasma*. What probably happened is that the second and third cows were normal, but their samples were contaminated in the collection process."

Uterine discharge on the udder — an especially common sight at calving — can spread *Mycoplasma* to the mammary system.



***Mycoplasma* organisms lack cell walls and are resistant to antibiotics in the mammary gland.**



Cows with active *Mycoplasma* intramammary infections shed the organism in massive quantities, causing positive cultures to show heavy growth.

Blackmer recommends re-culturing positive animals that appear healthy, to confirm their *Mycoplasma* status before taking further action. Similarly, Britten suggests physical examination and a CMT test for such animals, with another culture if they are CMT-negative. Most important, both practitioners emphasize the importance of carefully training the individuals taking the samples to reduce the incidence of contamination errors.

The other animals that present a quandary are known *Mycoplasma* cows that resolve the infection and remain productive. Rather than immediately culling these cows, Blackmer sometimes suggests permanently identifying and moving them into a segregated, "to-be-culled/do-not-breed/junk" string, along with cows that are confirmed positive but show no clinical signs. The main herd is then clean and pathogen-free, and the *Mycoplasma* cows can be milked until the end of their productivity, which may be years later.

Mycoplasma bacteria in pasteurized milk pose no known human health risk. Blackmer says cows that have survived one episode of *Mycoplasma* mastitis often endure and resolve subsequent flare-ups. No antibiotic or supportive therapy is needed, but if the clinical state becomes chronic or their productivity drops to a point of negative profitability, they should be culled.

This approach to temporarily salvaging *Mycoplasma*-positive animals may sound easy. It's not. Blackmer cautions that the individuals working with these cows must be thoroughly educated about the highly contagious nature of the disease and trained to protect the rest of the herd from contamination without fail. Steps in that process include:

- Double-identify *Mycoplasma*-positive cows with leg bands, ear tags, ear notches, etc.
- Milk the junk string at a designated sequence in the dairy's milking schedule, with thorough sanitation of the milking system — including internal

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Although more needs to be learned about the organism, one thing is certain: *Mycoplasma* mastitis is here to stay.

and external surfaces of clusters — after they pass through the parlor.

■ Never allow cows from the junk string to enter the hospital, where they could contaminate cows from the clean herd.

■ Never feed milk from known *Mycoplasma*-positive cows to calves, even if pasteurization is available. The risk of a breakdown in the process and subsequent infection of the calves is too great.

The decision of whether or not to temporarily keep *Mycoplasma*-positive cows on a dairy will vary from case to case and should be based on the dairy's economic condition; number of animals involved; level of milk production and reproductive status of infected animals; and the availability and price of replacements. Dairy managers also need to seriously consider whether they believe their staff and facilities can accommodate effective isolation and management of *Mycoplasma*-positive cows.

Hospital hot spot

In the throes of a severe *Mycoplasma* outbreak, other vigilant management efforts are needed. Clinical cows should be moved away from the rest of the herd to an isolation area. Blackmer prescribes a strict halt to anything going into any cow's teat end in the hospital — no mastitis tubes, no dilators, nothing.

"I am not a believer in the 'no intramammary therapy' approach to clinical mastitis management, but in the case of a *Mycoplasma* outbreak, I make an exception," says Blackmer. "The value of protecting *Mycoplasma*-negative cows in the hospital outweighs the benefit of treating them for other types of mastitis."

The practitioner describes some dairies' hospitals in the middle of an outbreak as "myco city." The heavy pathogen load that is shed from infected animals can quickly overwhelm the usually small hospital barn, to the point where virtually every exposed surface is contaminated. Blackmer suggests daily foam disinfection of the hospital's walls and other exposed surfaces to reduce the environmental presence of *Mycoplasma*.

If the hospital has its own milking facilities, he also recommends a thorough cluster-dunking process to disinfect milking units between every hospital cow.

Learning to live with it

Although more needs to be learned about the organism, one thing is certain: *Mycoplasma* mastitis is here to stay. Its highly contagious nature, ability to survive in the environment and massive pathogen loads shed from infected animals make it uniquely challenging to control. As it surfaces in new areas of the country, the disease will only add to the managerial mix in today's changing dairy industry.

"The penalty for error with this disease is pretty high," says Britten. "It behooves us all as veterinarians to get a good handle on how to manage it before we have to deal with a clinical outbreak."

Blackmer relates a *Mycoplasma*-education example often used by a former California extension agent. "He would stand in front of a room of veterinarians or dairyman holding up a vial containing no more than 10 mL of milk and say, 'In this vial I have enough *Mycoplasma* bacteria to infect every cow in California.'

"He was right, but the example may not have completely hit home for the listeners until they dealt personally with a *Mycoplasma* outbreak," Blackmer says. "Client compliance is much higher if a dairy producer has endured a bout with the disease. Unfortunately, a growing number of producers and veterinarians across the country are receiving crash courses in *Mycoplasma* management. It is not an impossible disease to control, but it's certainly one that should keep us all on our toes." ■

Westlaw.

1998 WL 1736155 (Bd.Pat.App & Interf.)
(Cite as: 1998 WL 1736155 (Bd.Pat.App & Interf.))

*1

Board of Patent Appeals and Interferences

Patent and Trademark Office (P.T.O.)
EX PARTE HERVY A. MORRIS
Appeal No. 98-2109
Application No. 08/500,315 [FN1]

NO DATE REFERENCE AVAILABLE FOR THIS DOCUMENT

Kokjer, Kircher, Bowman and Johnson

2414 Commerce Tower

911 Main Street

Kansas City, MO 64105-2088

Before CALVERT, NASE, and CRAWFORD

Administrative Patent Judges

CALVERT

Administrative Patent Judge

ON BRIEF

DECISION ON APPEAL

This is an appeal from the final rejection of claims 1 to 3, 5 and 9 to 11, the examiner having indicated in the answer that, as to the other claims remaining in the application, claims 21 to 24 are allowed, and claims 4, 20 and 25 would be allowable if rewritten in independent form.

The subject matter in issue is exemplified by claim 1, the only independent claim on appeal, which reads (emphasis added):

A liquid-jet cutting device comprising:

a cutting element for emitting a liquid-jet stream to cut a product located upon a product support surface;

an assembly for moving the cutting element between a cutting position located a cutting distance, within a cutting range, from the product and an idle position located an idle distance from the product support surface; and

a deflector disk, located proximate the idle position of the cutting element, to deflect the liquid-jet stream when the cutting element is moved to the idle position.

The reference applied in the final rejection is:

Driver 5,318,395 Jun. 7, 1994

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Claims 1 to 3, 5 and 9 to 11 stand finally rejected as being anticipated by Driver, under 35 U.S.C. § 102(b).

We note initially that on page 11 of the brief, appellant argues that Driver is nonanalogous art. This argument will be given no consideration, because it is well settled that "the question whether a reference is analogous art is irrelevant to whether that reference anticipates." In re Schreiber, 128 F.3d 1473, 1478, 44 USPQ2d 1429, 1432 (Fed. Cir. 1997).

The basis of the rejection is set forth on pages 3 and 4 of the final rejection (Paper No. 11).

Appellant argues that Driver does not anticipate claim 1 because there is no disclosure of the two limitations underlined in the copy of the claim, supra. With regard to the first of these limitations, i.e., the recitation "to cut a product located upon a product support surface," the examiner argued in the final rejection (page 7) that Driver meets this limitation because the pipeline 30 supports the product (liner) 35 to be cut. Appellant, on the other hand, asserts that this limitation must be interpreted "to be a surface which supports the product around where it is being cut," otherwise the limitation is superfluous (brief, page 7).

It is fundamental that during the examination of an application, the pending claims must be interpreted as broadly as their terms reasonably allow, and that limitations appearing in the specification may not be read into the claims. In re Zletz, 893 F.2d 319, 321, 13 USPQ2d 1320, 1322 (Fed. Cir. 1989); In re Prater, 415 F.2d 1393, 1404, 162 USPQ 541, 550 (CCPA 1969). Here, all that claim 1 specifies is that the product is "located upon a product support surface," and does not require that the product be supported at the point where it is being cut. Driver's liner 35, the product being cut, is located upon the surface of pipe 30 which supports it; therefore, interpretating the limitation in question as broadly as reasonable, it is met by Driver, even though Driver's liner 35 is not supported (i.e., backed up) by pipe 30 at the point where it is being cut by the liquid from nozzle 604. If this limitation is superfluous, as argued by appellant, that is simply an indication of its breadth.

***2** Turning to the second limitation in dispute, the examiner argues (answer, pages 5 and 6):

> [t]he phrase "to deflect the liquid-jet stream ....." should not be construed as defining structure. It does not describe any structure; it merely expresses what the disk is desired to do. However, it has well been established that, a recitation of the intended use of the claimed invention must result in a structural difference between the claimed invention and the prior art in order to patentably distinguish the claimed invention from the prior art. If the prior art structure is capable of performing the intended use, then it meets the claim. In re Casey, [370 F.2d 576, 580,] 152 USPQ 235[[, 238] (CCPA 1967); In re Otto, [312 F.2d 937, 940,] 136 USPQ 458, 459 (CCPA 1963). Therefore, it is irrelevant whether Driver's disk deflects fluid when the cutting element is moved to the idle position. However under certain conditions such as when Driver's device is inserted in a pipe section that already has been ported, Driver's disk will certainly deflect fluid when the cutter is moved to the idle position as there is no liner material between the cutting element and the disk to hinder the stream of fluid from hitting the disk.

Although we appreciate the examiner's position, we do not agree with his argument, because in our view the disk 620 of Driver is not capable of performing the intended use recited, i.e., of "deflect[ing] the liquid-jet stream when the cutting element is moved to the idle position." While the disk 620 is located

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"proximate the idle position" of cutting element (nozzle) 604, as claimed, it cannot perform the function of deflecting the jet from the nozzle 604 when the nozzle is in the idle position (Fig. 7), because, since nozzle 604 does not emit a jet when it is in that position there is no jet to be deflected; as disclosed by Driver at col. 5, lines 27 to 36 (see also claim 5), supplying fluid to cutter 600 will cause the nozzle 604 to move outward (from the Fig. 7 idle position) and contact the inside wall of the liner (Fig. 8). The examiner's statement in the last sentence of the above-quoted argument is not understood, since a stream of fluid is not emitted from Driver's nozzle 604 when it is in the idle position.

Our conclusion that claim 1 is not readable on the Driver apparatus is not contrary to the Otto or Casey decisions cited by the examiner. [FN2] Unlike those cases, the present limitation does not constitute "a method concept" which may not be relied on to distinguish a structural claim over the prior art (Otto, id.), or a manner or method of using the claimed machine "which is not germane to the issue of patentability of the machine itself" (Casey, id.). Rather, the limitation is in the nature of a structural limitation, in that it effectively requires a cutting element which is capable of emitting a liquid-jet stream when in the idle position; otherwise, there would be no stream for the disk to deflect. Driver does not disclose a cutting element which can operate in such a manner, and therefore does not anticipate claim 1. We note in this regard that "[t]here is nothing intrinsically wrong in defining something by what is does rather than by what it is." In re Echerd, 471, F.2d 632, 635, 176 USPQ 321, 322 (CCPA 1973).

***3** Accordingly, the rejection of claim 1, and therefore of claims 2, 3, 5 and 9 to 11 dependent thereon, will not be sustained.

Conclusion

The examiner's decision to reject claims 1 to 3, 5 and 9 to 11 is reversed.

No period for taking any subsequent action in connection with this appeal may be extended under 37 CFR § 1.136(a).

REVERSED

BOARD OF PATENT APPEALS AND INTERFERENCES

IAN A. CALVERT

Administrative Patent Judge

JEFFREY V. NASE

Administrative Patent Judge

MURRIEL E. CRAWFORD

Administrative Patent Judge

FN1. Application for patent filed July 10, 1995.

FN2. See also In re Schreiber, 128 F.3d at 1477, 44 USPQ2d at 1431 ("the recitation of a new intended use for an old product does not make a claim to that old product patentable").

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END OF DOCUMENT

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Research in Veterinary Science 1980, 29, 328–332

Effect of serial passages through liquid medium on the virulence of *Mycoplasma bovis* for the mouse mammary gland

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The mouse mastitis model was used to examine strains of *Mycoplasma bovis*. Strains that had been passaged in liquid medium more than 60 times were markedly less virulent than the same or different strains with fewer passages. Whereas the low passage strains produced a systemic response in some mice and severe pathological and histopathological changes in the mammary glands of all, the high passage strains produced only minor histopathological changes.

THE EXPERIMENTAL MOUSE mastitis model (Chandler 1970) has been used to study mycoplasma (Anderson *et al* 1976) and ureaplasma mastitis (Howard *et al* 1975) and was found to be a suitable animal model for investigation of these infections. Anderson *et al* (1976) found that a recent field strain of *Mycoplasma bovis* showed virulence for mice similar to that found in cattle. Furthermore the type strain of *M bovis* (NCTC 10131) did not multiply to the same extent or cause such a severe neutrophil response as the field strain.

The mouse model makes possible fuller studies of mammary infections by *M bovis* and a comparison of infections by various strains.

Materials and methods

Mycoplasma bovis *strains*

The type strain (NCTC 10131) and four Weybridge isolates, 5M192, 5M1171, 6M206 and 6M1170, from the lungs of calves were used. The Weybridge isolates were cloned at least three times and identified using the indirect fluorescent antibody test. All strains were grown in E broth containing 20 per cent horse serum, as described previously (Thorns and Boughton 1978) and incubated at 37°C. The number of viable organisms per ml was determined as colony forming units (CFU) on agar medium incubated in an atmosphere of about 7 per cent carbon dioxide in air. Serial passages were performed in E broth, subculturing every 48 to 72 h. Each passage represented approximately a 30-fold dilution of the inoculum.

Mice

Primiparous mice of the CFLP strain were used five to eight days after parturition. The inoculations were performed 1 to 2 h after the offspring had been removed from their mothers.

Mouse inoculation

Mice were inoculated in the fourth mammary glands from anterior on right and left sides (R4 and L4) as described by Chandler (1970) with 0·1 ml of E medium containing a known number of CFU of *M bovis*. A control group was inoculated with 0·1 ml of E medium only. Both the inoculated glands were removed at necropsy on the third day.

Necropsy procedures

Both the glands were halved, one half being fixed in 12 per cent neutral buffered formalin for histological examination. The other half was ground in 2·0 ml of E broth and the number of CFU in the homogenate determined, so that the number of CFU in the whole gland could be calculated. The homogenate was also spread onto sheep blood agar plates and incubated aerobically at 37°C to check for bacterial contamination.

The percentage of lactation tissue was calculated from histological sections by counting the number of points on a 100-point graticule at ×63 magnification that fell on either alveoli or blood vessels. Twenty-five fields were counted for each group of mice and an average was obtained (Anderson *et al* 1976).

Immunoperoxidase staining

To demonstrate mycoplasmas in the infected mammary glands sections were stained using a modification of the immunoperoxidase stain (Hill 1978). The paraffin embedded sections were passed through two changes of xylene (10 to 15 sec for each change) and three changes of 95 per cent ethanol (10 to 15 sec

for each change). All the slides were placed in 0·5 per cent hydrogen peroxide in methanol for 30 min to block any endogenous peroxidase activity. Preadsorption for 30 min in phosphate buffered saline (PBS), pH 7·2, containing 3 per cent normal goat serum was then carried out. After 5 min in buffer the sections were treated with an optimum dilution of antiserum (rabbit anti-*M bovis*) at room temperature for 30 min. The sections were washed in three changes of buffer for 10 min each wash, then treated with optimally diluted peroxidase conjugated with anti-rabbit IgG serum (prepared in goats, Miles Biochemicals) for 30 min and washed again with three changes of buffer for 10 min each wash. They were then treated with 0·05 per cent 3, 3-diaminobenzidine tetrahydrochloride (dissolved in 0·05 M tris, pH 7·6) and 0·01 per cent hydrogen peroxide for 5 min to stain the peroxidase. The slides were then washed in tap water and counterstained with methyl green for 5 min, dehydrated and mounted. Control slides were similarly treated but using normal rabbit serum.

Experimental design

To investigate the difference in virulence between high and low passage strains of *M bovis* groups of five mice were used. The first groups of mice were inoculated with strains passaged in broth no more than four times. Two groups were inoculated with strains passaged 33 or 61 times and four groups inoculated with strains passaged 91 or 138 times. A control group was inoculated with sterile E medium.

Results

Effects of inoculation of low passage strains of M bovis

When groups of mice were inoculated with low passage strains a systemic response was produced in one or two of the mice in each group (Table 1). These mice had ruffled coats and exophthalmia, and were hunched in appearance and disinclined to move. There was macroscopic evidence of inflammation of the mammary glands in all the mice. Glands were swollen and hard, with enlarged lymph nodes, and there was some extravasation of blood cells (Table 1). The histopathological changes in all the glands were severe, although only with strain 5M192 were more organisms recovered from the glands than were inoculated (Table 1). The neutrophil response in the ducts of glands inoculated with low passage strains was intense, most of the cells appearing pyknotic. There was a marked reduction in alveolar diameter compared with the control glands and some degenerative changes in the alveolar epithelium. There

TABLE 1: Mycoplasma strains inoculated into mouse mammary glands

M bovis strain	Number of passages	Inoculum per gland (log_{10})*	Number recovered per gland (log_{10})*	Percentage lactation tissue	Systemic response†	Pathology Gross‡	Pathology Histology§
5M 192	3	7·0	7·8	37·8	1R	5A	+ + + +
5M 1173	3	7·1	4·7	48·3	2R	5A	+ + + + +
6M 1170	3	6·2	4·6	46·5	1R	5A	+ + + + +
6M 206	4	6·0	4·3	41·8	1R	4A·1N	+ + + +
6M 206	33	7·3	5·2	65·8	|	4A·1N	+ + + +
6M 206	61	5·1	4·0	47·9	|	3A·2N	+ +
6M 206	91	7·0	3·0	58·7	|	1A·4N	
6M 206	91	6·1	2·0	43·9	|	1A·4N	+ + + +
5M 192	104	6·0	3·0	38·0	|	5N	+ + + +
NCTC 10131	138	6·1	3·3	60·5	|	5N	1
5M 192				66·3	|	5N	
Control	-						

* Geometric mean of 10 glands from five mice
† R Number of mice in the group with systemic signs of disease
‡ A number of mice in the group with macroscopically inflamed glands
N Number of mice in the group with normal glands
§ + Neutrophil response, majority morphologically normal; + + intense neutrophil response, majority morphologically normal; + + + neutrophil response, majority pyknotic; + + + + intense neutrophil response, majority pyknotic

FIG 1: Mouse mammary gland three days after inoculation with *M bovis* strain 5M192 passaged three times in E broth. The alveolar architecture has been obscured due to perialveolar cell infiltration. Giemsa × 400

was also a mild infiltration of mononuclear cells in the perialveolar area and interalveolar fat, obscuring the alveolar architecture (Fig 1). There was a small reduction in lactation tissue compared with the glands inoculated with sterile medium (Table 1).

Effects of inoculation of strains subcultured 33 *and* 61 *times in E medium*

Strain 6M206 passaged 33 and 61 times failed to produce a systemic response in any of the mice, but

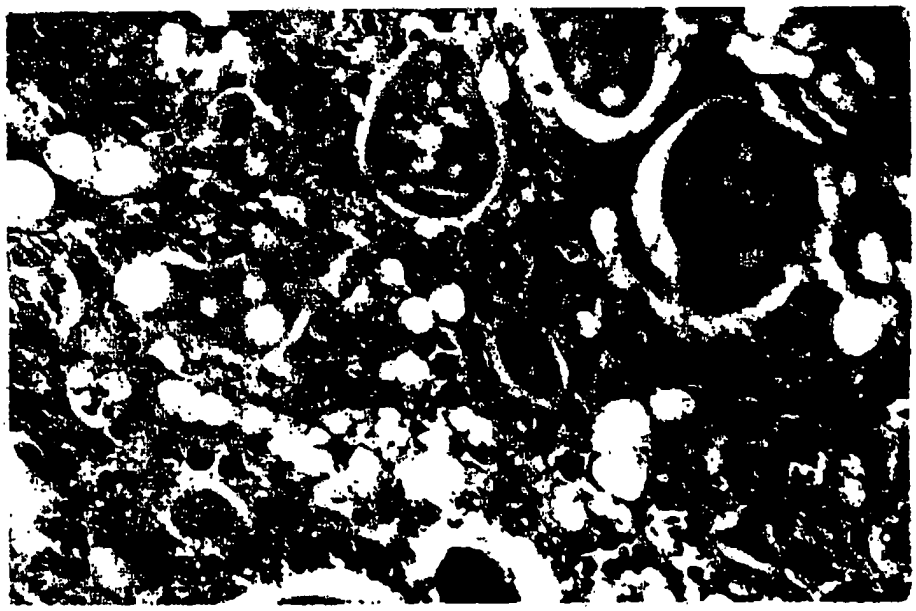

FIG 2: Mouse mammary gland three days after inoculation with *M bovis* strain 5M192 passaged 138 times in E broth. The alveoli are normal containing milk products and their architecture is clearly visible. Giemsa × 400

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FIG 3: Mammary gland of mouse three days after inoculation with E broth. The alveoli are large and there is no neutrophil response. Giemsa ×400

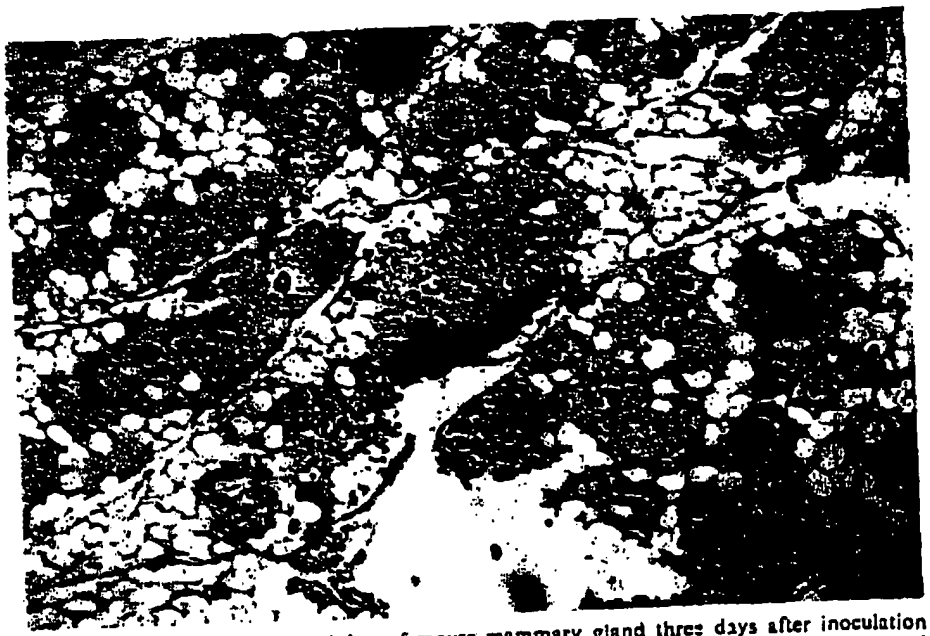

FIG 4: Immunoperoxidase staining of mouse mammary gland three days after inoculation with *M bovis* strain 5M192 passaged three times in E broth. The alveolar architecture is obscured and discrete microcolonies can be seen associated with cell membranes. ×400

glands from four and three mice respectively showed macroscopical evidence of inflammation with a neutrophil response in the ducts and larger glands (Table 1). The number of organisms recovered was similar to that recovered from low passage strains.

Effects of inoculation of high passage strains

The three high passage strains (91 to 138 passages) did not produce a systemic response in any of the mice, and glands from only one mouse in each of

two groups showed signs of inflammation (Table 1). The glands from the rest of the mice were white and full of milk, similar in appearance to the control glands. Where a neutrophil response occurred it was mild, affecting only the larger ducts, although macrophages were seen in the perialveolar area. The alveoli were normal, many full of milk products, and their architecture was clearly visible (Fig 2). Compared with the low passage strains fewer organisms were recovered. The percentage of lactation tissue varied, but in two cases there was no involution relative to the control glands.

Effects of inoculation of sterile E broth

The glands inoculated with E medium showed no neutrophil response and most of the alveoli were full of milk products (Fig 3).

Immunoperoxidase staining

Mycoplasmas localised in discrete microcolonies apparently associated with cell membranes (Fig 4) were present in all inoculated glands. No peroxidase staining was seen in any of the control uninoculated glands.

Discussion

The four low passage strains of *M bovis* all produced a severe inflammatory response in the mammary glands. The histopathology of the infected glands was similar to that previously reported (Anderson *et al* 1976) for *M bovis* infections. There are no previous reports of a systemic response produced by inoculating mice with ureaplasmas or mycoplasmas (Howard *et al* 1975; Anderson *et al* 1976), but a number of generalised reactions were found when low passage strains were used in these experiments. That these reactions were due to bacterial invasion seems unlikely as the mammary glands were free from bacterial infection. Although Howard *et al* (1975) found no differences between strains of mice in the severity of ureaplasma mastitis, the possibility that our strain of mice may be more sensitive to mycoplasma infection cannot be ruled out.

When strains were passaged more than 90 times their virulence for the mouse mammary gland was reduced. In most cases the neutrophil response was reduced so that there was no gross evidence of inflammation and, if present, it was mild with no degenerative changes in the epithelium of the alveoli and ducts. Anderson *et al* (1976) found marked differences in virulence between the avirulent vaccine strain KH_3J and the highly virulent Gladysdale strain of *M mycoides* subsp *mycoides* and to a lesser extent differences between *M bovis* strain NCTC 10131 and strain Ab/1, a recent isolate. We were able to show differences in virulence as marked as the differences between the two strains of *M mycoides* subsp *mycoides*. It seems therefore that strains passaged many times in liquid medium lose their virulence for the mouse mammary gland.

Immunoperoxidase staining showed the organisms existing in discrete microcolonies throughout the mammary gland, apparently associated with cell membranes. Kehoe *et al* (1967) found similar microcolonies in the mammary glands of cattle and from their results postulated that a soluble factor, possibly a haemolysin, was involved in pathogenesis. However, Mosher *et al* (1968) and Bennett and Jasper (1978a, b, c) postulate immunological mechanisms whereby interactions between the organisms and antibody may cause tissue damage. Bennett and Jasper (1978a, b, c) have shown a temporary defect in cellular and humoral immunity early in infection when cattle have been infected with *M bovis*. It is possible that the avirulent strains are therefore being cleared from the tissues by immune mechanisms.

Whatever mechanisms the virulent strains have lost or modified, they should provide further insight into the pathogenesis of *M bovis* mastitis which could perhaps lead to a stable vaccine for this disease.

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veterinary microbiology

Genomic, protein and antigenic variability of *mycoplasma bovis*

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(Accepted 21 September 1993)

Abstract

Restriction endonuclease analysis (REA) with three enzymes *Sma*I, *Pst*I, *Bam*HI- was used to identify 13 different genomic groups among 37 *Mycoplasma bovis* strains. One genomic group was comprised of 14 strains. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns for one strain chosen from each genomic group and an international reference strain PG45 were all similar. Antigenic variability in *M. bovis* species was investigated by immunoblotting, using serum from a calf that had been naturally infected with *M. bovis* and three *M. bovis*-specific monoclonal antibodies – mAbs N_2, I_2 and 5D7. Twenty *M. bovis* field strains were tested, comprising one from each genomic group, six from the same genomic group and the reference strain. Antigenic profiles obtained with calf serum differed markedly one from the other, the heterogeneity being equally great among the strains belonging to the same genomic group as those coming from different groups. A stable antigen common to 164 out of 168 strains was detected by mAb N_2, whilst with mAbs I_2 and 5D7, two different membrane antigenic systems were demonstrated that were strikingly variable. These variations in expression occurred not only from one strain to another, but also within the same lineage of clones from a single cell.

Keywords: Mycoplasma bovis; Variability, antigenic, genomic; Monoclonal antibody;

1. Introduction

Mycoplasma (M.) *bovis* has an important role in many bovine diseases. It has been shown experimentally to be pathogenic for the respiratory system (Gourlay et al., 1976,

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Gourlay and Houghton 1985), for joints (Pfutzner et al., 1983, Chima et al., 1980, Gourlay et al., 1976, Stalheim and Page, 1975), for the mammary gland (Bennett and Jasper, 1978, Horvath et al., 1983) and for the reproductive system (Stalheim and Proctor, 1976, Bocklisch et al., 1986). *M. bovis* occurs in cattle all over the world (Erno and Perreau, 1985) causing extensive economic losses (Thomas and Jasper, 1982, Jasper, 1982, Pfutzner, 1984, Gourlay et al., 1989). In France, *M. bovis* is often the cause of respiratory diseases in calves (Poumarat et al., 1988, 1991).

The control of *M. bovis*-related diseases would be greatly assisted if a specific, sensitive and rapid diagnostic technique could be developed, capable of detecting all *M. bovis* strains. By using monoclonal antibodies (Heller et al., 1992, Boothby et al., 1986) or nucleic acid probes (Demuth et al., 1992, McCully and Broch, 1992, Mattsson et al., 1991), it should be possible to improve substantially both specificity and rapidity of diagnostic methods. However, if very specific reagents were used, it could result in detection of only some of the known strains, particularly if there should be a degree of variability among *M. bovis* strains. This question of variability has received little attention, although Sachse et al. (1992) demonstrated that there was notable similarity between the protein patterns of 34 *M. bovis* strains.

The present study was set up to investigate the genomic, protein and antigenic variability of *M. bovis*, by comparing 37 isolates using restriction endonuclease analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with polyclonal or monoclonal antibodies (mAbs).

2. Materials and methods

2.1. *M. bovis* strains

Thirty six isolates recovered in France and international reference strain PG45 (Hale et al., 1962) were studied. The geographic origin and isolation date of these strains together with the associated pathology are presented in Table 1. One hundred and seventy other *M. bovis* strains were used to test the universal applicability of three mAbs for detecting *M. bovis*. All strains were identified as *M. bovis* by membrane filtration immunoblotting (MF dot) (Poumarat et al., 1991). They gave a specific reaction with rabbit hyperimmune serum prepared against PG45 strain. They were cloned three times as described by Tully (1983).

2.2. *Growth medium: generation of clonal lineages*

Organisms were grown in modified PPLO broth (DIFCO) (see Poumarat et al., 1991) with 1% PPLO serum fraction (DIFCO) replacing the 20% horse serum.

For the preparation of subclones of *M. bovis* strain 1067, culture in late log phase was filtered through a 0.2 μm membrane (Millipore) and serially diluted to 10^{-6}. 0.1 ml of each dilution was spread evenly over the surface of agar plates, which were incubated for 4 days at 37°C. Several well-isolated colonies (first clone series) were picked and inoculated into 2 ml of broth. These cultures were in turn incubated and when growing actively, were dispersed in 15 ml of broth. After further incubation the cultures were prepared for electro-

Table 1
Thirtyseven strains of *M. bovis* tested in this study

Number	Isolation date	Geographic origin	Pathology
PG45 (I.r.s.)	1962	Unknown	Unknown
1067	1983	Manche	Mastitic mammary gland
2008	1984	Unknown	Unknown
2011	1984	Côtes d'Armor	Arthritis
2012	1984	Creuse	Pneumonic lung
2021	1984	Corrèze	Pneumonic lung
2025	1984	Mayenne	Pneumonic lung
2028	1984	Loire (Haute)	Pneumonic lung
2036	1982	Meuse	Unknown
2037	1984	Lozère	Pneumonic lung
2049	1982	Finistère	Pneumonic lung
2064	1986	Isère	Pneumonic lung
2088	1985	Ain	Pneumonic lung
2163	1978	Isère	Pneumonic lung
2171	1978	Isère	Pneumonic lung
2193	1986	Ile et Vilaine	Pneumonic lung
2234	1984	Aveyron	Pneumonic lung
2259	1985	Saône et Loire	Metritis
2283	1985	Loire Atlantique	Pneumonic lung
2317	1987	Ile et Vilaine	Unknown
2480	1987	Maine et Loire	Pneumonic lung
3279	1988	Italy	Pneumonic lung
3322	1988	Corrèze	Pneumonic lung
3339	1988	Somme	Pneumonic lung
3478	1988	Mayenne	Pneumonic lung
3668	1989	Vienne (Haute)	Unknown
3731	1989	Tarn et Garonne	Unknown
3876	1989	Mayenne	Pneumonic lung
3882	1989	Tarn et garonne	Pneumonic lung
3919	1989	Morbihan	Pneumonic lung
4277	1989	Ain	Pneumonic lung
4568	1990	Maine et Loire	Pneumonic lung
4785	1990	Calvados	Abscess
4904	1990	Aveyron	Unknown
4906	1990	Mayenne	Pneumonic lung
4964	1991	Mayenne	Pneumonic lung
5113	1991	Aveyron	Pneumonic lung

I.r.s. = International reference strain (PG45).

phoresis and immunoblotting with mAbs I_2 et 5D7 as described below. Using the same method as already described, a second clone series was produced from one clone of the first clone series and so on for the third, fourth, fifth and sixth series. In all, 48 clones were tested – six from each of the first four series and 12 from the fifth and from the sixth series.

2.3. Preparation of extracts

The cultures of each strain were harvested in late log phase by centrifugation at 13000 *g* for 35 min at 4°C. For whole cell protein analysis, pellets were washed three times in tris

buffered saline solution (TBS) (Tris HCl, 0.01 M; NaCl, 0.15 M; pH, 7.2) and then once in tris buffered solution (Tris HCl, 0.125 M; pH, 6.8).

The protein content was estimated (see Lowry et al., 1951) and the pellets were then frozen at +70°C for future use. For DNA analysis, the pellets were washed twice in tris EDTA, EGTA buffered solution (TEEB) (Tris HCl, 0.02 M; pH, 8; EDTA, 0.01 M; EGTA, 0.002 M) and also frozen at −70°C.

2.4. TX 114 phase partitioning

Triton X 114 (TX 114) phase partitioning was carried out using the procedure of Bordier (1981) adapted for mycoplasma. The mycoplasmas were suspended in TBS containing 1% w/v TX 114 and phenylmethylsulfonylfluoride (0.001 M) and incubated at 4°C for 30 min. The preparations were centrifuged at 10000 *g* for 5 min at 4°C to remove insoluble material. The supernatants were transferred to new tubes and subjected to three cycles of phase fractionation. The final detergent and aqueous phases were adjusted to 2% w/v TX 114 ready for SDS-PAGE analysis.

2.5. DNA purification

The method described by Razin et al. (1983a) was used to purify DNA. The DNA precipitate was dried *in vacuo* and the pellet dissolved in 110 μl of TEEB. DNA concentration was determined by measuring absorbance at 260 nm.

2.6. Digestion of DNA with restriction endonucleases

10 μg of mycoplasma DNA were digested by the restriction endonucleases *Pst*I, *Bam*HI or *Sma*I, following the manufacturer's instructions (Boehringer Mannheim). 1 μg of phage lambda DNA was digested as a control for each enzyme activity. The digested DNA was submitted to electrophoresis for 3 h, stained with ethidium bromide (0.4 μg/ml) for 15 min and photographed under UV-light. For each strain a control preparation of undigested DNA was submitted to electrophoresis to detect possible extra chromosomal DNA; none was detected.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

SDS-PAGE was performed following the procedure described by Laemmli (1970) with 8–22% separating gel. Whole cell proteins and molecular standard mass preparations (SIGMA) were dissolved in sample standard buffer (Tris HCl, 0.05 M; pH, 6.8; SDS, 2.5%; 2-mercaptoethanol, 5%; glycerol, 15%; bromophenol blue, 0.05%) and heated in a water bath (100°C) for 5 min. Insoluble material was removed by centrifugation at 10000 *g* for 10 min. Solubilized cell proteins and standards were submitted to electrophoresis at

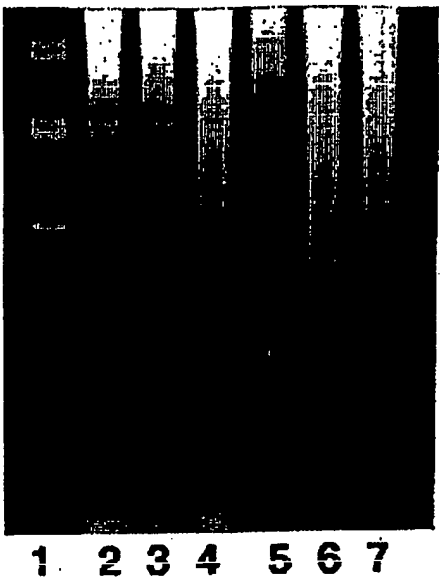

Fig. 1. Five different (3 to 7) *PstI* restriction endonuclease patterns obtained from the 37 *M. bovis* strains tested. Tracks 1, Lambda digested DNA; 2, reference strain PG45; 3, strain 2036; 4, strain 3876; 5, strain 2088; 6, strain 3339; 7, strain 4568.

Table 2
Classification of the 37 *M. bovis* strains according to their restriction enzyme cleavage patterns obtained with *SmaI*, *PstI*, *BamHI*

Genomic group number	Restriction enzyme cleavage patterns			*M. bovis* strains numbers
	SmaI	*PstI*	*BamHI*	
1	1	1	1	n = 14: PG45, 1067, 2008, 2012, 2021, 2036, 2037, 2064, 2163, 2193, 2480, 3322, 3668, 3882
2	1	2	1	n = 2: 3279, 3919
3	1	3	2	n = 4: 2088, 2283, 2317, 4906
4	2	2	3	n = 5: 2025, 2028, 2171, 2234, 2259
5	2	1	4	n = 2: 4904, 4964
6	2	5	3	n = 2: 3731, 4568
7	3	1	4	n = 2: 4277, 4785
8	2	1	3	n = 1: 5113
9	2	4	3	n = 1: 2011
10	2	4	5	n = 1: 3339
11	3	1	3	n = 1: 2049
12	3	2	3	n = 1: 3876
13	x	4	4	n = 1: 3478

constant current of 20 mA per gel at 15°C until the dye front had migrated to the end of the gel.

2.8. Immunoblotting

Transfer

Proteins were transferred from gel to nitrocellulose membrane filter with pore size 0.45 μm (Schleicher and Shuell) in a transfer unit (Hoeffer) with a current of 600 mA for 90 min at 13°C. (Electrode buffer: Tris HCl, 0.025 M; glycine, 0.192 M; pH, 8.3; methanol, 15%; SDS, 0.1%).

Serum and monoclonal antibodies

The polyclonal calf serum J008, used in the present study, was selected from sera obtained from calves that had been infected by contact with calves that had been experimentally inoculated via the respiratory tract with *M. bovis* strain 1067. Prior to this experiment, the

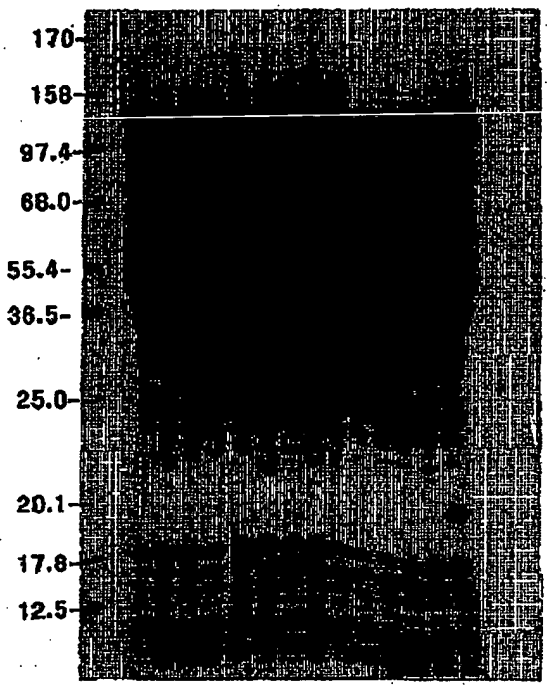

Fig. 2. SDS-polyacrylamide gel electrophoretic (gradient 8 to 22 %) patterns of whole cell proteins from 14 *M. bovis* strains (one for each of the 13 genomic groups and reference strain PG45) (80 μg protein loaded per track). From left to right: reference strain PG45, 2012, 3279, 2088, 2171, 4964, 4768, 4785, 5113, 3339, 2049, 3876, 3478, 2011. (Positions of molecular mass standards (kilodaltons) are indicated on the left).

calves had been shown to be free of all *M. bovis* infection. The J008 serum titre was 320 with indirect hemagglutination (Cho et al., 1976, Poumarat et al., 1987).

Three specific *M. bovis* mAbs were used – mAbs N_2 et I_2 prepared from *M. bovis* strain 1067 (supplied by Vétoquinol Biotechnologie) and mAb 5D7 prepared from international strain PG45 (supplied by Istituto Zooprofilattico Sperimentale, Brescia).

Immunoassay

Once the proteins had been transferred to the nitrocellulose membrane, they were blocked on the sheet by incubating for 1 h with a solution of 10% horse serum in TBS. The sheet was then washed three times with TBS containing 0.05% Tween 20 and once with TBS only, each washing lasting for 3 min.

Sheets were then incubated for 2 h at 35°C with each of the four primary antibodies diluted in 5% horse serum in TBS. Calf serum J008 was diluted to 1/200, *mAb*I_2 to 1/1500, mAb N_2 1/1000, and mAb 5D7 to 1/330.

The sheets were then washed exactly as before and then incubated for 1 h at 35°C with

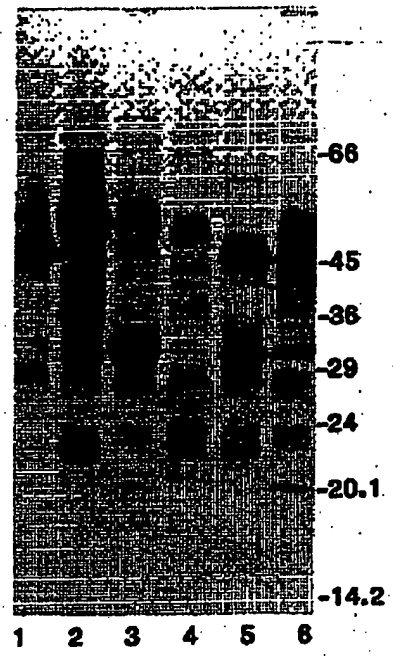

Fig. 3. Immunoblots of whole cell proteins of 6 *M. bovis* strains, belonging to 6 different genomic groups, obtained with calf serum J008 from calf infected by *M. bovis* 1067 (160 μg protein loaded per track). When medium was similarly probed no antigens were detected. Tracks 1, strain 3478; 2, strain 4964; 3, strain 2049; 4, strain 5113; 5, strain 3279; 6, homologous strain 1067. (Positions of molecular mass standards (kilodaltons) are indicated on the right).

the corresponding peroxidase-labelled secondary antibody diluted in the 5% horse serum. Once again the sheets were washed as before and then treated with developing solution (TBS containing one part per thousand of a 5% (w/v) solution of 4-chloro-1-naphtol in methanol and one part per ten thousand of hydrogen peroxide). When the colour reaction was complete, further reaction was stopped by washing with distilled water.

3. Results

3.1. Restriction endonuclease analysis

The 37 *M. bovis* strains studied gave five different electrophoretic patterns with *Bam*HI, four with *Sma*I and five with *Pst*I (Fig. 1). Based on the combination of the different electrophoretic profiles obtained with the three enzymes, the 37 strains could be classified in 13 genomic groups (Table 2) In group 1 there were 14 strains including the international

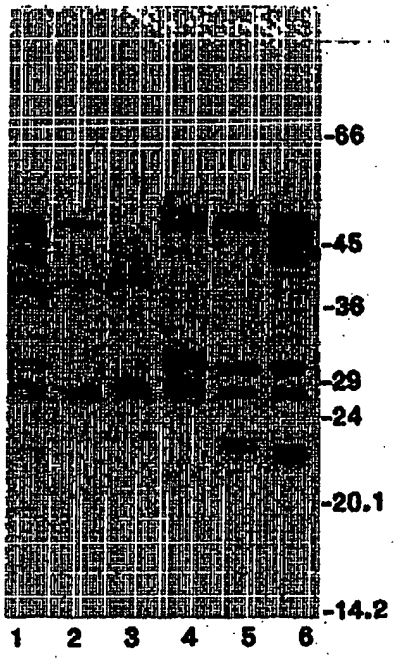

Fig. 4. Immunoblots of whole cell proteins of 6 *M. bovis* strains belonging to the same genomic group (n°1) obtained with calf serum J008 from calf infected by *M. bovis* 1067 (160 μg protein loaded per track). When medium was similary probed no antigens were detected. Tracks 1, homologous strain 1067; 2, reference strain PG45; 3, strain 2036; 4, strain 3668; 5, strain 3882; 6, strain 3322. (Positions of molecular mass standards (kilodaltons) are indicated on the right).

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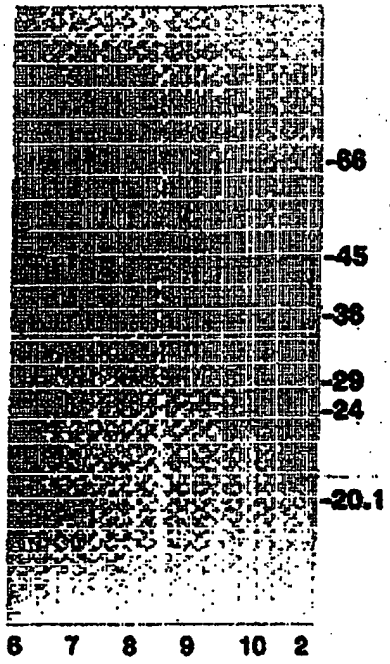

Fig. 5. Immunoblots of whole cell proteins of 6 *M. bovis* strains, belonging to 6 different genomic groups, obtained with monoclonal antibody N_2 (160 μg protein loaded per track). Tracks 6, strain 1067; 7, reference strain PG45; 8, strain 3478; 9, strain 5113; 10, strain 3339; 2, strain 3785. (Positions of molecular mass standards (kilodaltons) are indicated on the right).

reference strain PG45. There appeared to be no significant relationship between genomic and geographic distribution nor between the genomic distribution and isolation dates, nor yet between the genomic distribution and the type of pathology associated with the strains.

There was, however, a statistically significant difference in the heterogeneity of strains isolated before 1987, where 18 strains fell into five groups, with nine strains in group 1, compared with the 19 strains isolated after 1987, which fell into 10 groups with four strains in group 1.

3.2. Whole cell protein analysis by SDS-PAGE

The electrophoretic patterns of one strain for each of the 13 genomic groups and of strain PG45 were compared (Fig. 2). Except for a few minor differences in band sites and intensity, the patterns of all the strains showing some 60 different polypeptides were similar.

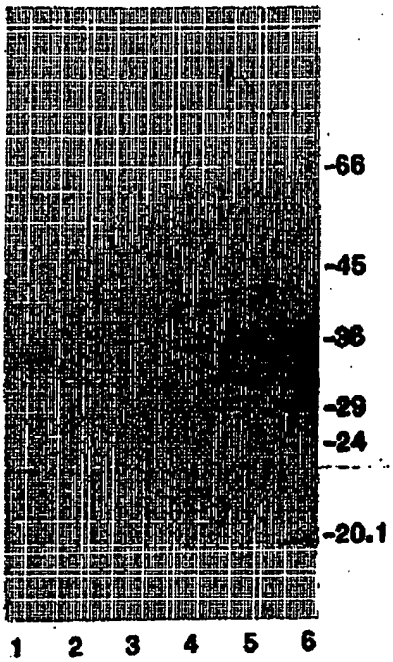

Fig. 6. Whole cell proteins immunoblots of six *M. bovis* strains from six different genomic groups obtained with monoclonal antibody I_2 (160 μg protein loaded per track) Tracks 1, 2012; 2, 4785; 3, 4964; 4, 2171; 5, 2028; 6, 1067. (Positions of molecular mass standards (kilodaltons) are indicated on the right).

3.3. Antigenic analysis by immunoblotting

Immunoblots using calf serum J008 were obtained from one strain for each of the 13 genomic groups, for strain PG45 and for 6 strains from genomic group 1 (Figs. 3 and 4). The differences were striking both between the patterns from the 13 genomic groups and from those in group 1 and these were most significant in the regions 66–36 and 24–20 kDa.

When compared with the profile of the immunoblot from homologous strain 1067, the number and position of bands are seen to vary from one strain to another and some bands are seen to be reactive in the profiles of some of the heterologous strains, that do not exist at all in the profile of strain 1067.

When immunoblots were obtained from the same series of *M. bovis* strains, but using the three monoclonal antibodies N_2, I_2 and 5D7, the results were more complex. mAb N_2 showed the presence of one protein at about 45 kDa that was present in all strains except strain 5113 (Fig. 5). Using the MF dot test, mAb N_2 was shown to react with all except four out of the 168 other *M. bovis* strains that were tested (2.4%).

The immunoblot for homologous strain 1067 using *mAb*I_2 showed 11 bands with molec-

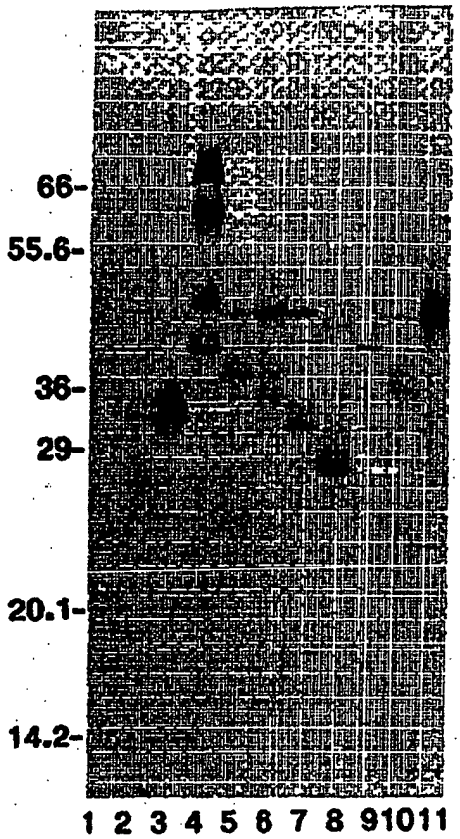

Fig. 7. Whole cell proteins immunoblots of 11 *M. bovis* strains, (one for each of 11 different genomic groups), obtained with monoclonal antibody 3D7 (10 μg protein loaded per track). Tracks 1, strain 3478; 2, 3876; 3, 2049; 4, 3339; 5, 5113; 6, 4785; 7, 4568; 8, 2025; 9, 4906; 10, 3919; 11, reference strain PG45. (Positions of molecular mass standards (kilodaltons) are indicated on the left).

ular weights ranging from 54–20 kDa, with five major bands at 54, 36, 29, 22 and 19 kDa. The comparison with five heterologous strains, each from a different genomic group is shown in Fig. 6. With this monoclonal antibody, the patterns from the various strains were strikingly different, whether they came from the same genomic group or not. The patterns varied from one strain to another, both in the number and in the position of the bands. Strains PG45 and 2036, for example, showed only one band, whilst for strain 3478 there was only an extremely narrow band at 20 kDa. Using the MF dot test, *mAbI*$_2$ was shown to react only weakly or not at all with 27 strains out of 103 other *M. bovis* strains that were tested (26%).

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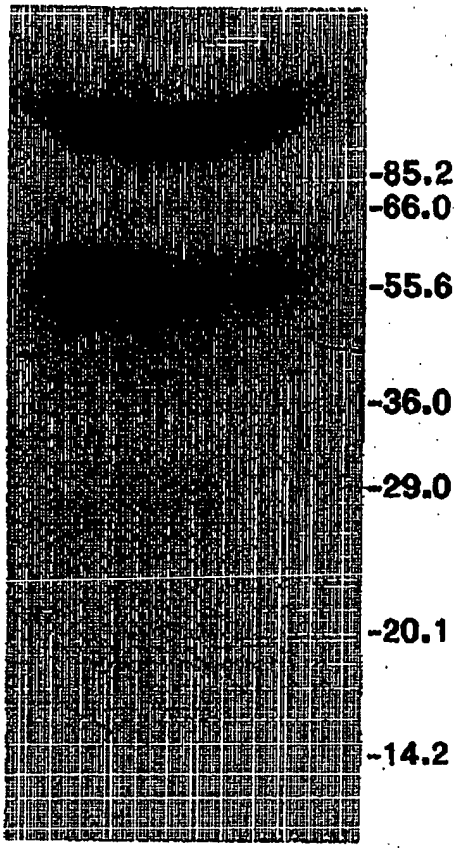

Fig. 8. Examples of the more important variations among immunoblots of 48 subclones of strain 1067 obtained with monoclonal antibody 5D7 (10 μg protein loaded per track). (Positions of molecular mass standards (kilodaltons) are indicated on the right).

Using mAb 5D7 against strains belonging to 11 different genomic groups, there was again striking variation between strains. Strain PG45 showed many bands between 25 and 85 kDa. Strain 3478 showed no bands at all (Fig. 7). When seven strains from genomic group 1 were tested, the patterns were again strikingly different, except for one strain which was similar to strain PG45. Using the MF dot test, mAb 5D7 was shown to react with all except seven out of the 91 other *M. bovis* strains that were tested (8%).

Six successive generations of strain 1067 in 48 subclones were tested using mAb 5D7. The immunoblots of the subclones varied in number, position and intensity of bands, with

the extreme example of one subclone that, within the limits of the test, did not express the antigen at all (Fig. 8).

When the same strain 1067 subclones were challenged with $mAbI_2$, there was variation only in the intensity of the bands in the immunoblots.

Immunoblots obtained with N_2, I_2 or 5D7, either from whole cell proteins of strain 1067, or from the cell protein phase partitionating with TX 114 were absolutely identical, indicating that the antigens detected by these three mAbs were included in the cell membrane.

4. Discussion

4.1. Restriction endonuclease analysis (REA)

The idea of using cleavage patterns of the mycoplasmic genome as an aid to taxonomy was first suggested by Bove and Saillard (1979). Since then, REA has been widely used, particularly for epidemiological studies. REA patterns of individual isolates were highly reproducible and remained unchanged even after long-term passages (Kleven et al., 1988a, Ionas et al., 1991a). Depending on the mycoplasma species, the degree of variability within the species may be greater or less. *M. ovipneumoniae* species, for example, is very heterogeneous (Ionas et al., 1991a,b) whereas *M. pneumoniae* is homogeneous (Razin et al., 1983b, Chandler et al., 1982). Results may vary, depending on the sampling of strains and on the choice of enzymes (Su et al., 1991, Chandler et al., 1982). For a study to be complete and precise, a wide range of strains must be selected and tested by several endonucleases. Before strain variability can be firmly established, at least two enzymes need to be used, to avoid errors due to DNA methylation which could modify enzyme activity (Razin et al., 1983a, International Committee, 1988).

For the present study, a number of different enzymes were considered and *SmaI*, *PstI* and *Bam*HI were selected, since they cleave *M. bovis* DNA in relatively few sites facilitating interpretation. Out of the 37 *M. bovis* strains tested with these three enzymes, 14 strains including PG45 were shown to be identical, but the remaining 23 strains appeared to be very heterogeneous, being distributed among 12 different genomic groups, each of which was characterized by its specific restriction enzyme cleavage pattern (Table 2). There were therefore 13 genomic groups in all. Were more strains and especially recent isolates, to be tested, the number of different genomic groups would certainly increase.

When other species of mycoplasma, such as *M. gallisepticum*, were tested, the REA pattern appeared to be correlated with the geographical origin of the strains or with the animal host (Kleven et al., 1988a,b, Santha et al., 1988, Kleven, 1990), but for *M. bovis* there was no correlation between the genetic heterogeneity of the strains and their geographic origin, their isolation date, or the associated pathology. Moreover, the heterogeneity was more marked among the strains most recently isolated.

4.2. Protein and antigenic analysis

One *M. bovis* strain was selected from each of the 13 genomic groups and the protein patterns were obtained by SDS-PAGE. The similarity of the patterns, matched with the

results obtained on 34 *M. bovis* strains carried out by Sachse et al., (1992), but the comparison could be no more than approximate, because of the larger number – about 60 – of separate polypeptides. Using numerical analysis (Costas et al., 1987), it would be possible to quantify precisely the degree of similarity between the strains, but in any case it should be borne in mind that mycoplasmas, such as *M. hominis* or *M. arthritidis*, which appear to be homogeneous when tested by the 1D-PAGE, showed some variability when tested by the more discriminating 2D-PAGE technique (Watson et al. 1988, Stadlander and Watson, 1992).

Antigenic profiles of the *M. bovis* strains obtained by immunoblotting with J008 calf serum differed markedly one from the other, the heterogeneity being equally great among strains belonging to the same genomic group and those coming from different genomic groups. There appeared to be no relation between the genomic variability of *M. bovis* and the antigenic variability, unlike that observed for other Mollicutes such as *Ureaplasma* (Watson et al., 1990). The heterogeneity found here is in contrast to the homogeneity reported by Sachse et al., (1992) who studied a less diversified batch of strains in which, nevertheless, they reported important differences for at least two strains.

Mycoplasma species, when submitted to metabolism and growth inhibition tests, have shown antigenic variations (*M. gallisepticum*, Duplellet et al., 1990 and *M. ovipneumoniae*, Jones et al., 1976), that have never been reported for *M. bovis*, which, in contrast, appears to exhibit a global antigenicity that is fairly homogeneous (Poumarat et al., 1992). Both this and the protein patterns appear to be in contradiction with the great variability of antigenic patterns revealed by immunoblotting. However, similar phenomena have already been reported for *M. hominis* (Andersen et al., 1987), *M. arthritidis* (Stadlander and Watson, 1992) and for *M. gallisepticum* (Avakian et al., 1991).

There could be two explanations for the observed variability of the immunoblots. Firstly, there could be a real difference between strains, with some strains not exhibiting certain antigens. With a library of mAbs prepared from one strain of a species, it may be possible only to recognize some of the strains of that same species (Panangala et al., 1992, Thirkell et al., 1990, Landfoged et al., 1990). The antigen revealed by mAb N_2 displayed this kind of variability. Secondly, the variability could result from variations in the molecular weight of the antigen from one strain to another. This has been shown for *M. fermentans* (Stadlander et al., 1991), for *M. arthritidis* (Stadlander and Watson 1992), for *Ureaplasma urealyticum* (Watson et al., 1988), *M. hyorhinis* (Boyer and Wise 1989) and *M. pulmonis* (Watson et al., 1988).

There may also be the case where both explanations are valid at the same time. Recently, an unusual system of antigenic variation was characterized in *M. hyorhinis* (Rosengarten and Wise, 1991) and also in *M. pulmonis* (Watson et al., 1988). Variant lipoproteins (vlps), expressed on the membrane surface appear to undergo high frequency phase variations both in expression and size. These variations are random, appearing not only between two strains of the same species, but even within the same lineage of clones from a single cell.

mAbI_2 and 5D7 displayed two different membrane antigenic systems with characteristics similar to those of the vlps: their localization on the membrane and their extreme variability in expression and size, could not, however, be related to any genetic or epidemiological

factor. Moreover, the degree of variation within clones of a single cell depended on the system (I_2 or 5D7).

The present study showed that great heterogenity exists within the species *M. bovis*, so that, before any new method of diagnosis – either direct or indirect – can be proposed, it is essential to evaluate all the specific reagents used in the test, on a large number of diverse strains. This applies equally to mAbs, DNA probes and PCR primers.

Two highly variable antigenic systems have been identified so far for *M. bovis*, but their in vivo function is not known. It is evident that this antigenic variability must be taken into account in developing diagnostic and vaccination strategies. Further studies will be necessary to determine whether other variable antigenic systems are likely to exist in the species *M. bovis* and whether major *M. bovis* antigens are involved.

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Experimental Intramammary Inoculation with *Mycoplasma bovis* in Vaccinated and Unvaccinated Cows: Effect on Milk Production and Milk Quality

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ABSTRACT

The effect of vaccination on milk production was evaluated in vaccinated and control cows experimentally challenged in two of four quarters with live *Mycoplasma bovis*. During the first three weeks after experimental challenge, six of eight unchallenged quarters on vaccinated cows and seven of eight unchallenged quarters on control cows became infected. Most of these quarters secreted normal milk, with negative California Mastitis Test scores and maintained normal milk production throughout most of the study (although some quarters on trol cows remained infected). All llenged quarters became infected, had strong California Mastitis Test reactions, and had a drastic (> 85%) loss in milk production. Thereafter, four of eight challenged quarters on control cows remained infected, had mostly positive California Mastitis Test scores, produced mostly normal-appearing milk, and recovered some productive capabilities. By the end of the study no *M. bovis* could be recovered from challenged quarters on vaccinated cows and the milk appeared mostly normal. The California Mastitis Test scores on these quarters, however, remained elevated and milk production remained very low.

Key words: Mastitis, *Mycoplasma bovis*, vaccination, milk production, milk quality.

RESUMÉ

Cette expérience visait à évaluer l'effet de la vaccination sur la production lactée, chez des vaches vaccinées et témoins, soumises à une infection de défi avec *Mycoplasma bovis* dans deux des quartiers de leur pis. Au cours des trois semaines ultérieures à cette infection, six des huit quartiers non infectés des vaches vaccinées et sept des huit quartiers non infectés des vaches témoins le devinrent. La plupart de ces quartiers sécrétaient du lait normal, qui réagissait de façon négative au "California Mastitis Test", et ils affichèrent une production normale, tout au long de l'expérience, quoique certains quartiers des vaches témoins demeurèrent infectés. Tous les quartiers soumis à l'infection de défi demeurèrent infectés, réagirent fortement au test précité et affichèrent une baisse d'au delà de 85% de leur production. Après cela, quatre des huit quartiers des vaches témoins, soumis à l'infection de défi, demeurèrent infectés, réagirent surtout de façon positive au test précité, produisirent surtout du lait normal et améliorèrent sensiblement leur capacité sécrétrice. À la fin de l'expérience, on ne pouvait plus isoler *M. bovis*, des quartiers des vaches vaccinées, soumis à l'infection de défi, et leur lait était surtout normal. La réaction du lait de ces quartiers au test précité demeura élevée et leur production resta très basse.

Mots clés: mammite, *Mycoplasma bovis*, vaccination, production lactée, qualité du lait.

INTRODUCTION

Mycoplasma bovis is responsible for the most common and severe form of mycoplasmal mastitis (1,2). The prevalence of *M. bovis* udder infections appears to be increasing, and it has been isolated from animals in a number of major dairy-producing areas in this country and elsewhere (1-4). Since antimicrobial therapy is ineffective in controlling mycoplasmal disease (1,3-5), development of an effective prophylactic vaccine is an attractive possibility for controlling *M. bovis* mastitis.

Although the nature of immunological resistance to *M. bovis* mastitis remains unclear, several authors have observed resistance in previously infected cows or herds (3,6,7) and there are reports of improving resistance to *M. bovis* disease by prophylactic vaccination (8,9,10). If prophylactic vaccination is to be efficacious, it must have minimal effects on the health and productive capabilities of the cow. While neither mortality nor prolonged morbidity are commonly associated with *M. bovis* mastitis the quality and amount of milk produced by previously infected quarters can be quite variable (3). To our knowledge, there are no reports to date on the effect of vaccination with *M. bovis* on milk production. The present study was designed to evaluate milk produc-

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tion and milk quality in vaccinated and control cows during experimental intramammary challenge exposure with live *M. bovis*.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Eight Holstein cows in late lactation with confirmed conception dates within three weeks of one another were obtained from a local commercial dairy farm with no prior history of mycoplasmal disease. The cows were transported to the university facility where they were managed essentially as on the farm of origin. While lactating, the cows were fed alfalfa hay *ad libitum* and a dairy grain ration in excess of nutritional requirements. Cows were milked twice daily using strict pre- and postmilking hygiene, employing an organic iodine-detergent preparation demonstrated effective against mycoplasmas (11). The milking machine was specially designed and manufactured to prevent quarter to quarter transfer of milk and/or air, and for quantitation of quarter milk production.

The cows were monitored for one week before milking was stopped eight weeks prior to their expected parturition date. During this time the cows showed no clinical mastitis, produced normal milk with negative California Mastitis Test (CMT) scores (12), had low serum *M. bovis*-specific IgG reactivity as measured by enzyme-linked immunosorbent assay (ELISA) (13), and had negative milk cultures for mycoplasma and bacteria (14). After the last milking (week 1) the cows were infused with 1 x 10^6 IU penicillin in 10 mL saline in each quarter.

Experimental treatments and observations were made on all the cows at the same time each day or week (Fig. 1). Weeks were numbered from 0-20, beginning one week after the cows arrived at our facility. Parturition times for the cows were on experimental weeks 7, 10, 11 and 11 for the vaccinated cows, and 7, 9, 9 and 10 for the control cows. Intramammary challenge exposure was performed one week after all the cows had calved (week 12).

TABLE I. The Experimental Scheme for the Four Quarters on the Four Vaccinated Cows (*M. bovis*) and the Four Quarters on the Four Control Cows (placebo) for Quarter Vaccination (Q, weeks 6 and 8) and Experimental Challenge Exposure (C, week 12)

		Quarter Designation			
		-/-	-/C	Q/-	Q/C
Cow Number	1	A[a]	B	C	D
	2	B	D	A	C
	3	D	C	B	A
	4	C	A	D	B
		n = 4	n = 4	n = 4	n = 4

		Quarter Designation	
		Challenged	Unchallenged
Cow Number	1	AC	BD
	2	BA	DC
	3	DB	CA
	4	CD	AB
		n = 8	n = 8

-/- = Not vaccinated/not challenged, -/C = not vaccinated/challenged, Q/- = vaccinated/not challenged, Q/C = vaccinated/challenged

[a]A = right front, B = right rear, C = left front, D = left rear

EXPERIMENTAL DESIGN

Four experimental cows were selected and numbered 1-4 for vaccination and the remaining four cows were selected and numbered 1-4 for controls using a random number table. The four vaccinated cows received killed *M. bovis* antigen systemically (subcutaneously) and locally in designated quarters by intramammary infusion (Table I). A latin squares design was used for designating local quarter vaccination (either real or sham) and experimental challenge exposure so that orthogonality could be established between estimates of the various effects (Table I). The four control cows received sham vaccination consisting of phosphate buffered saline using the same volumes, schedule, and procedures. All eight cows were experimentally challenge exposed by intrammary infusion with live *M. bovis* in quarters designated by latin square design. The only randomization was with respect to cow group (vaccinate or control) and numbers 1-4 (treatment designation).

Analysis of milk production and milk quality variables showed no difference between unchallenged quarters which had been locally vaccinated (either real or sham) and those which had not been locally vaccinated. Similarly there was no difference in these variables among locally vaccinated and unvaccinated quarters which were challenge exposed to *M. bovis*. Therefore, the original latin square was collapsed on the local vaccination category and a simplified analysis comparing unchallenged and challenged quarters on systemically vaccinated or control cows was pursued (Table I B). Note

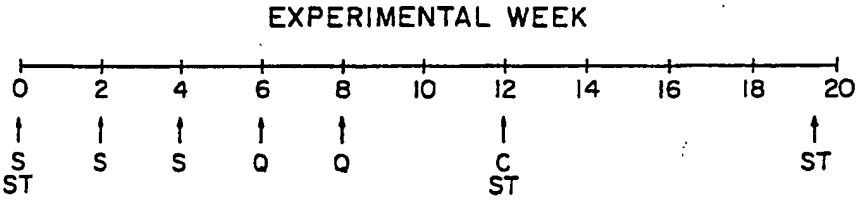

Fig. 1. The systemic (S) and quarter (Q) vaccine, skin test (ST) and challenge exposure (C) schedule for four vaccinated (killed *M. bovis*) and four control (placebo) cows observed for 19.5 weeks. (S = systemic vaccination, Q = quarter vaccination, C = experimental challenge, ST = skin test).

that the orthagonality of the design was maintained (15).

VACCINATION

Mycoplasma bovis strain California 201 was used for preparation of vaccine and skin test antigen and also for experimental challenge exposure (Fig. 1). The serological and biochemical relationships of this isolate, with other strains and method of preparation, have been reported (16-18).

The four vaccinated cows were inoculated with 2 mL of antigen (1 mg formalin killed *M. bovis* protein) in Freud's complete adjuvant at three locations subcutaneously at weeks 0, 2 and 4. Local vaccination was done with 3 mL (1 mg formalin killed *M. bovis* protein) in designated quarters by intramammary infusion at weeks 6 and 8. These cows were also skin tested at weeks 0, 12 and 19 with 0.1 mg *M. bovis* protein in 0.2 mL at three locations as previously described (18,19). The four control cows were given a similar placebo inoculation (without *M. bovis* protein) at the same times, but were skin tested with *M. bovis* antigen as above at week 19. All eight cows were challenged in designated quarters with 1.5×10^6 cfu live *M. bovis* in phosphate buffered saline containing 20% fetal calf serum at week 12.

MILK PRODUCTION

Quarter milk production for each cow was measured in liters for two daily milkings. The mean milk production for three days (six milkings) was calculated for each quarter on each cow at week 12 before challenge and this value was defined as 100% production. Similar calculations for the quarters were made for each subsequent time period and expressed as a percentage relative to week 12 (100%).

MILK QUALITY

A visual test tube examination of milk quality was made on each quarter milk sample at each time period and graded on appearance as normal, watery, flaky, discolored, or purulent according to the specifications of the Coliform Mastitis Research Committee (20).

CALIFORNIA MASTITIS TEST

A cowside CMT (12) was performed on every quarter at the morning milking. A detectable CMT (0.5, 1, 2 or 3) at any milking for each time period (3 days) was scored as positive for that quarter and week and a CMT of 0 was scored as negative.

MICROBIOLOGICAL CULTURE

Each quarter on each cow was cultured for the presence of mycoplasmas and the major bacterial pathogens at each time period by standard methods (14). Negative (< 10 cfu/mL) and positive (≥ 10 cfu/mL) mycoplasma culture results were scored accordingly.

RESULTS

MICROBIOLOGICAL CULTURE

All experimentally challenged quarters became infected and most (six of eight among vaccinates and seven of eight among controls) unchallenged quarters became infected. At the end of the study four of eight challenged and two of eight unchallenged quarters on three of four control cows remained infected, and no mycoplasmas could be recovered from challenged or unchallenged quarters on vaccinated cows. No major bacterial mastitis pathogens were recovered at any time.

SKIN HYPERSENSITIVITY

Some slight immediate skin hypersensitivity in all cows existed prior to vaccination. A strong immediate and delayed hypersensitivity was present in vaccinated cows prior to challenge at week 12. Challenge infection at week 12 resulted in immediate and delayed hypersensitivity in control cows at week 19 similar to that of cows which were both vaccinated and challenged.

CALIFORNIA MASTITIS TEST

All experimentally challenged quarters developed CMT reactivity. All challenged quarters on vaccinated cows remained CMT-positive for the duration of the study while some challenged quarters on control cows became CMT-negative. Among

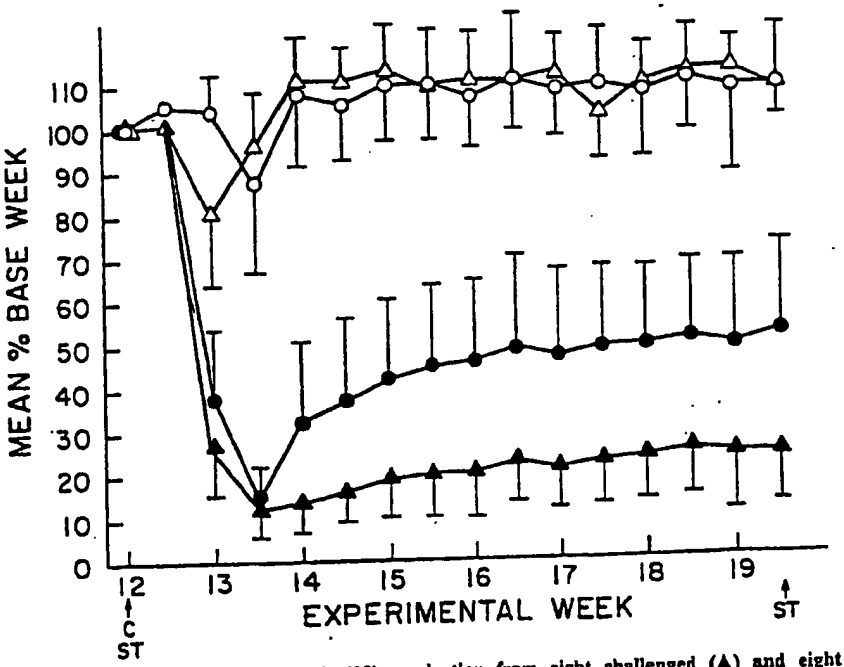

Fig. 2. Mean percent of base week (12) production from eight challenged (▲) and eight unchallenged (△) quarters on four vaccinated cows and eight challenged (●) and eight unchallenged (○) quarters on four control cows experimentally challenge exposed by intramammary infusion with live *M. bovis* and monitored for 7.5 weeks (weeks 12-19.5). (C = challenge exposure, ST = skin test).

unchallenged quarters, four of eight quarters on vaccinated cows and three of eight quarters on control cows developed CMT reactivity, but most became CMT-negative by the end of the study.

MILK PRODUCTION IN UNCHALLENGED QUARTERS (Fig. 2)

The mean milk production during 128 unchallenged quarter-observations on the four control cows was above 100% at all times except week 13.5 at which time three of the eight quarters had a mean production of 87% (Fig. 2). The mean milk production during 128 unchallenged quarter-observations on the four vaccinated cows was less than 100% at only two observation times (weeks 13 and 13.5).

MILK PRODUCTION IN CHALLENGED QUARTERS (Fig.2)

Mean milk production during 128 challenged quarter-observations on the four control cows dropped severely to below 50% at week 13, and to 15.4% at week 13.5. At the time of lowest production (week 13.5) all eight challenged quarters on all four control cows were producing less than 30% of their initial production level. Thereafter, the mean production of this group recovered gradually to about 50% production by week 16.5, with two quarters producing in the 60-90% range and six quarters producing in the 30-60% range most of the time.

Mean milk production during 128 challenged quarter-observations on the four vaccinated cows dropped severely to less than 30% at week 13 and to less than 15% at week 13.5. Thereafter, the production in this group remained the lowest of any group observed, maintaining a production level in the 15-20% range most of the time. After week 13 production of six or more of the eight quarters in this group was less than 30% of initial levels.

MILK QUALITY IN UNCHALLENGED QUARTERS

Milk quality evaluations from unchallenged quarters from the four vaccinated and four control cows indicate that normal-appearing milk can be produced from quarters which are infected with *M. bovis* and have an increased cell content as indicated by the CMT.

Unchallenged quarters on the four control cows produced normal-appearing milk 96.7% of the time with only four samples detected which were watery or flaky. Despite the production of normal-appearing milk most of the time, 46 of 120 (38.3%) of the quarter samples contained *M. bovis* and 36 of 128 (28.1%) had a detectable CMT reaction.

Unchallenged quarters on the four vaccinated cows produced normal-appearing milk 97.5% of the time with only three of the 120 samples being flaky or watery. Mycoplasma infection was detected in eight (6.7%) quarter samples and 14 (10.9%) CMT positive samples were observed.

MILK QUALITY IN CHALLENGED QUARTERS

Challenged quarters on the four control cows produced normal-appearing milk 70% of the time with a purulent secretion noted on five (4.23%) occasions. Despite production of normal-appearing milk most of the time, 77 of 120 (64.2%) of the samples contained *M. bovis* and 98 of 128 (76.6%) had a detectable CMT reaction.

Challenged quarters on the four vaccinated cows produced normal-appearing milk 65.8% of the time, with a purulent secretion noted on 14 (10.9%) occasions. Fifty-seven of 120 (47.5%) samples contained *M. bovis* and 112 of 128 (87.5%) had a detectable CMT reaction.

DISCUSSION

Bovine mastitis reduces milk yield, changes milk composition, and is one of the major disease problems concerning the dairy industry (3,4). Systematic descriptions of milk production and milk quality during *M. bovis* infection, were important parameters in evaluating vaccine efficacy.

During the acute phase of the experimental *M. bovis* infection (weeks 12-15), all unchallenged quarters demonstrated only a slight, transient drop in production during which time some abnormal milk was observed, whereas challenged quarters from all animals experienced a drastic drop in milk production accompanied by abnormal and sometimes purulent lacteal secretions. The inflammation response occurred sooner and was more marked in vaccinates than in controls.

Unchallenged quarters from vaccinated and control cows which became infected with *M. bovis* and had a measurable CMT reaction could produce milk of normal appearance and quantity. This agrees with other reports of both natural and experimental infections (3,21,22). The participation of glandular compensation either in terms of milk production or endogenous inflammatory elements was not evaluated (23-25).

The return to normal milk production and appearance after natural and experimental *M. bovis* infection is quite variable (3). In a description of two of four cows experimentally inoculated with *M. bovis*, Bennett and Jasper (21) observed that both cows produced abnormal milk from post-inoculation (PI) day 5 in challenged quarters and from days 15-20 in unchallenged quarters. One cow continued to produce abnormal milk, high CMT scores, and remain agalactic for the duration of the experiment (PI = 56), while the other cow partially returned to normal production, and produced visually normal milk in all quarters by PI day 53. In two other experiments the same workers observed that only one quarter in one of four cows experimentally inoculated with *M. bovis* produced abnormal milk (22), while 10 of 11 (one quarter not done) quarters on three different cows which had experienced either natural or experimental *M. bovis* mastitis produced abnormal milk when repeatedly challenge exposed (7).

Other reports on milk production among cows or herds indicate that the recovery to normal milk production after the acute phase of *M. bovis* mastitis varies from a few weeks to poor performance and agalactia even in subsequent lactations (3). The mechanism by which *M. bovis* causes mastitis is undocumented, but the severity of the mastitis has been related to the stage of lactation and infection, and the severity and acuteness of the inflammatory

response, as well as to prior sensitizing exposure to specific antigens (3).

The involvement of prior sensitization to mycoplasma antigens in adverse reactions to subsequent exposure to live organisms has been observed in a number of species. Many factors such as the mycoplasma strains, host species and the individuality of responses among members of the same species, and method of sensitization and experimental challenge exposure undoubtedly affect the nature and degree of adverse reactions. Since *M. bovis* is a common inhabitant of the bovine respiratory tract of calves with and without signs of respiratory disease, and naturally infected calves can develop measurable specific immune responses, the nature of prior sensitization with *M. bovis* antigens is difficult to document on cows experiencing *M. bovis* mastitis. The role of prior sensitization in the severity of *M. bovis* mastitis, and its potentially adverse effect on milk production and milk quality appears to be an important parameter in evaluation of vaccine efficacy, and in need of further clarification.

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